BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/00, A01H 1/00 — A1

(11) International Publication Number: WO 93/02187

(43) International Publication Date: 4 February 1993 (04.02.93)

(21) International Application Number: PCT/US92/05786

(22) International Filing Date: 13 July 1992 (13.07.92)

(30) Priority data:
732,243 19 July 1991 (19.07.91) US

(71) Applicant: MICHIGAN STATE UNIVERSITY [US/US]; E. Lansing, MI 48824 (US).

(72) Inventors: SOMERVILLE, Christopher, Roland ; 4596 Herron Road, Okemos, MI 48864 (US). POIRIER, Yves ; 704 Cherry Lane, E. Lansing, MI 48824 (US). DENNIS, Douglas, Edward ; Route 2, Box 92A, Weyers Cave, VA 24486 (US).

(74) Agent: REED, David, T.; The Procter & Gamble Company, Ivorydale Technical Center, 5299 Spring Grove Avenue, Cincinnati, OH 45217-1087 (US).

(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, RO, RU, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: TRANSGENIC PLANTS PRODUCING POLYHYDROXYALKANOATES

(57) Abstract

The present invention relates to transgenic plants which produce poly-beta-D-hydroxybutyric acid (PHB) and related polyhydroxyalkanoates (PHA). The production of PHB is accomplished by genetically transforming the plants with modified genes from microorganisms. The genes encode the enzymes required to synthesise PHB from acetyl-CoA or related metabolites. PHB is a very useful polymer which is biodegradable.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

TRANSGENIC PLANTS PRODUCING POLYHYDROXYALKANOATES

Field of the Invention

This invention concerns the introduction and expression of certain genetic informational material, i.e., DNA which includes genes coding for proteinaceous materials and/or genes regulating or otherwise influencing the production thereof, into cells of higher plants and the regeneration of fertile plants from the genetically transformed cells. The purpose of this genetic intervention is to transfer to higher plants, from microbial organisms, the ability to synthesize polymeric materials composed of linear polyesters of hydroxy acids. This class of materials is generally referred to as polyhydroxyalkanoates. The specific example shown here is the production of polyhydroxybutyrate (PHB).

BACKGROUND OF THE INVENTION

Many species of bacteria accumulate granules of polyesters composed of hydroxyacyl monomers which serve as carbon reserves. The occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates has recently been reviewed (Anderson, A. and Dawes, E. A., Microbiol. Rev. 54:450-472 (1990)). The most commonly found compound of this class is poly(D(-)-3-hydroxybutyrate). However, some species accumulate copolymers of different hydroxyalkanoates such as 3-hydroxypentaneoate (Wallen, L. L. and Rohwedder, W. K., Environ. Sci. Technol. 8:576-579 (1974)). At least 11 short-chain 3-hydroxyacids are found as components of polymers from marine sediments. Studies of polyhydroxyalkanoate production in Alcaligenes eutrophus have shown that when the bacteria are cultivated in a

medium with only glucose as a carbon source, only PHB is accumulated. However, when both glucose and propionic acid are provided as carbon sources, the bacteria accumulates random copolymers of 3-hydroxypentanoate and 3-hydroxybutyrate (Holmes, P. A., Phys. Technol. 16:32-36 (1985); Holmes, P. A., Wright, L. F. and Collins, S. H. European Patents 0 069 497, January 1983 and 0 052 459, December 1985). In addition, when A. eutrophus is supplied with various other carbon sources, polyesters containing 4-hydroxybutyrate and 5-hydroxyvalerate monomers are produced (Table I in Anderson, A. J. and Dawes, A. E., Microbiol. Rev. 54:450-472 (1990)). Thus, it appears that the composition of the polymer is regulated to some extent by the availability of alternative substrates for the enzymes which catalyzed synthesis of the polymer from monomers.

PHB accumulates in bacterial cells as granules of approximately 0.24 to 0.5 μm in diameter. On the basis of measurements of the molecular weight of PHB monomers, each granule has been estimated to contain a minimum of 1,000 polymer chains. The granules have been proposed to possess a membrane coat composed of lipid and protein representing approximately 0.5 and 2%, respectively, of the granule weight (Anderson, A. and Dawes, E. A., Microbiol. Rev. 54:450-472 (1990)). The activity of the PHB synthase enzyme is thought to be associated with this membrane. The state of the PHB within the granule is a matter of substantial uncertainty. Recent evidence suggests that the polymer within the granules is in an amorphous state. It is not known what regulates the size of PHB granules in any organism.

In most organisms, PHB is synthesized from acetyl coenzyme A (acetyl-CoA) by a sequence of three reactions catalyzed by 3-ketothiolase (acetyl-CoA acetyltransferase; EC 2.3.1.9), acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase; EC 1.1.1.36) and

poly(3-hydroxybutyrate)synthase. The pathway is shown in Figure 1. In Rhodospirillum rubrum, PHB is synthesized by conversion of L(+)-3-hydroxybutyryl-CoA to crotonyl-CoA to D(-)-3-hydroxybutyryl-CoA. The 3-ketothiolase has been purified from various PHB-synthesizing bacteria and has been studied in several species of higher plants. The role of the enzyme in higher plants is thought to be in the production of acetoacetyl-CoA for mevalonate production as well as in the degradation of fatty acids. The acetoacetyl-CoA reductase has been detected in a number of PHB-synthesizing bacteria. Several species, including A. eutrophus, appear to have two isoenzymes which differ with respect to substrate specificities and cofactor requirements. The NADH reductase of A. eutrophus is active with C4 to C10 D(-)- and L(+)-3-hydroxyacyl-CoAs, whereas the NADPH reductase is active with only C4 and C5 D(-)-3-hydroxyacyl-CoAs. An enzyme of this kind has never been reported in higher plants. PHB synthase activity has been detected in PHB-accumulating bacteria as both a soluble enzyme and as a granule-bound activity, depending on the growth conditions. Both forms of the enzyme have been partially purified but have not as yet been purified to homogeneity because of instability. The PHB synthases of A. eutrophus is specific for D(-)-enantiomers and when tested with 3-hydroxyacyl-CoAs, was shown to be active only with C4 and C5 substrates, consistent with the observation that only C4 and C5 3-hydroxyacid monomer units are incorporated into the polymer by this organism. The mechanism of PHB synthase action remains obscure. It is presumed that the chain transfer role played by the synthase must in some way control the molecular weight of the polymer produced, which is characteristic of a given organism. PHB synthase activity has never been reported in any plant.

Several groups of researchers have independently cloned, and expressed in E. coli, the genes involved in the biosynthesis of PHB by A. eutrophus (Slater, S. C., et al.,

J. Bacteriol. 170:4431-4436 (1988); Schubert, P., et al., J. Bacteriol. 170:5837-5847 (1988)). Recombinant strains of E. coli carrying a 5.2 kbp fragment from A. eutrophus were capable of accumulating substantial quantities of PHB as intracellular granules. The nucleotide sequence of the 5.2 kbp fragment was also independently determined by two groups (Janes, B. B., et al., In Dawes, E. A. (ed) Novel Biodegradable Microbial Polymers, Kluwer Academic Publishers, pp 175-190 (1990); Peoples, O. P. and Sinskey, A. J., J. Biol. Chem. 264:15293-15297 (1989); Peoples, O. P. and Sinskey, A. J., J. Biol. Chem. 264:15298-15303 (1989)). Analysis of the deduced amino acid sequences of the open reading frames, in conjunction with evidence based on genetic complementation studies, revealed that the 5.2 kbp fragment contained three closely linked genes encoding the three enzymes required for PHB production. A patent concerning the use of the cloned genes to overproduce the biosynthetic enzymes in bacteria has been filed (Peoples, O. P. and Sinskey, A. J., Int Patent WO 89/00202, January 1989).

Certain species of bacteria have the ability to excrete enzymes and degrade PHB and related polyhydroxyalkanoates (Reviewed in Anderson, A. and Dawes, E. A., Microbiol. Rev. 54:450-472 (1990)). Because of the prevalence of these bacterial species in many natural environments, PHB is rapidly degraded in soil and activated sludge. Thus, PHB and related polyhydroxyalkanoates are of interest as renewable sources of biodegradable thermoplastic. Industrial PHB production from large-scale cultivation of bacteria began in 1982. The PHB produced in this way is marketed by ICI plc under the trade name Biopol. However, because of the costs associated with growing and harvesting large cultures of bacteria, the PHB is much more costly to produce than polymeric materials such as starch which are accumulated to high levels in many species of higher plants. Therefore, it may be advantageous to

develop, by genetic engineering, lines of higher plants which accumulate PHB.

BRIEF DESCRIPTION OF FIGURES AND TABLES

Figure 1 shows the biochemical pathway for the production of polyhydroxybutyrate (PHB). In A. eutrophus, PHB is produced by the successive action of three enzymes: 3-ketothiolase, converting acetyl-CoA to acetoacetyl-CoA; acetoacetyl-CoA reductase, converting acetoacetyl-CoA to D(-)-3-hydroxybutyryl-CoA; PHB synthase, converting D(-1-3-hydroxybutyryl-CoA to polyhydroxybutyrate. In plants and animals, acetoacetyl-CoA is a precursor in the production of mevalonate.

Figure 2 shows the nucleotide sequence of the PHB operon from A. eutrophus. The sequence was obtained from Janes, B., Hollar, J. and Dennis, D. in Dawes, E. A. (ed), Novel Biodegradable Polymers, Kluwer Academic Publishers, 175-190 (1990). The open reading frame from nucleotide 842 to 2611 encodes the PHB synthase (phbC gene) (amino acids S1 to S589). The open reading frame from nucleotide 2696 to 3877 encodes the enzyme 3-ketothiolase (phb A gene) (amino acid T1 to T393). The open reading frame from nucleotide 3952 to 4692 encodes the enzyme acetoacetyl-CoA reductase (phb B gene) (amino acid R1 to R246). Underlined are the sequences for the restriction enzymes DdeI, BstBI, PstI, SacI and TthlllI. These restriction enzymes were used in the subcloning of the phb genes.

Figure 3 shows a schematic summary of the steps involved in the construction of plasmids pUC-THIO and pBI-THIO. The purpose of this latter plasmid is to place the 3-ketothiolase gene from A. eutrophus under transcriptional control of the CaMV 35S promoter so that it will be transcribed in higher plants. The top diagram represent the A. eutrophus PHB operon with the approximate location of the open reading frames encoding the PHB synthase, 3-ketothiolase and acetoacetyl-CoA reductase. The horizontal arrows indicate the direction of

transcription. The bottom diagram indicates the major components of the pBI121-derived plasmids: NPT II, neomycin phosphotransferase II gene encoding kanamycin resistance; CaMV 35S, cauliflower mosaic virus 35S promoter; poly A, polyadenylation sequence; RB, right border sequence of T-DNA; LB, left border sequence of T-DNA. The bottom diagram is not drawn to scale. Abbreviations for restriction enzyme sites: D, DdeI; P, PstI; B, BstBI; T, TthlllI; BH, BamHI; S, SacI; H, HindIII.

Figure 4 shows a schematic summary of the steps involved in construction of plasmid pUC-SYN and pBI-SYN. The purpose of this latter plasmid is to place the PHB synthase gene from A. eutrophus under transcriptional control of the CaMV 35S promoter so that it will be transcribed in higher plants. Diagrams and abbreviations are described in Figure 3.

Figure 5 shows a schematic summary of the steps involved in the construction of plasmids pUC-RED and pBI-RED. The purpose of this latter plasmid is to place the acetoactyl-CoA reductase gene from A. eutrophus under transcriptional control of the CaMV 35S promoter so that it will be expressed in higher plants. The top and bottom diagrams and abbreviations are described in Figure 3. The middle diagram is an enlargement of the acetoacetyl-CoA reductase gene region. The location and sequence of the PCR primer #1 and #2 are indicated. The last nucleotide at the 3' end of PCR primer #1 corresponds to nucleotide 3952 in Figure 2 and is the first nucleotide of the initiation codon for the reductase gene. The last nucleotide at the 3' end of PCR primer #2 is complementary to nucleotide 4708 in Figure 2. The additional BamHI and KpnI restriction enzyme sites created by the PCR primers are indicated.

Figure 6 shows Southern blot analysis of untransformed control and transgenic A. thaliana plants. One g of genomic DNA from untransformed A. thaliana race Rschew and from transgenic plants were digested with the restriction enzyme HindIII, the fragments were separated by

agarose gel electrophoresis and transferred to nylon membranes. Filters were hybridized to ^{32}P-labeled DNA fragments from genes (A) phbA, (B) phbB and (C) phbC. The genomic DNAs analyzed are: wild type A. thaliana race Rschew (lane a) and transgenics T4-3A (lane b), T3-2A (lane c), T4-2A (lane d), T4-3B (lane e), RedB-2G (lane f), RedB-2B (lane g), RedB-2E (lane h), RedB-2C (lane i), RedD-3A (lane j), RedB-2A (lane k), RedB-2D (lane l), S12-3A (lane m), S8-1-2C (lane n), S8-1-2A (lane o) and S8PUC-2B (lane p). Numbers on the left side are length in kilobase pairs.

Figure 7 shows Northern blot analysis of untransformed control and transgenic A. thaliana plants. Total RNA from wild type A. thaliana race Rschew (10 µg) and from transgenic plants (20 µg) were resolved by electrophoresis in formaldehyde-containing agarose gels and transferred to nylon membranes. Filters were hybridized to ^{32}P-labeled DNA fragments from genes (A) phbA, (B) phbC and (C) phbB. The RNAs analyzed are from plants: T3-2A (lane a), T4-2A (lane b), T4-3B (lane c), T4-3A (lane d), wild type A. thaliana (lanes e, j and r), S8PUC-2B (lane f), S8-1-2C (lane g), S12-3A (lane h), S8-1-2A (lane i), RedB-2D (lane k), RedB-2E (lane l), RedB-2G (lane m), RedB-2A (lane n), RedB-2B (lane o), RedB-2C (lane p) and RedD-3A (lane q). Numbers are length in kilobase pairs.

Figure 8 shows gas chromatography (GC) of purified PHB and plant extracts. GC spectra of transesterified chloroform extracts of leaves from untransformed wild type A. thaliana race Rschew (B) and F1 hybrid between transgenic plants S8-1-2A and RedB-2C (C) were compared to the chromatogram of transesterified commercial PHB (A). The arrows indicate the location of the ethyl-hydroxybutyrate peak.

Figure 9 shows gas chromatography-mass spectrometry analysis of ethyl-hydroxybutyrate prepared from a PHB standard and PHB from plant extracts. (A) Mass spectrum of transesterified commercial PHB; (B) the mass

spectrum of the GC peak from leaf chloroform extract of F1 hybrid between S8-1-2A and RedB-2C having a retention time identical to ethyl-hydroxybutyrate (as shown in Figure 8C).

Figure 10 shows transmission electron micrographs (TEM) of leaf and seed of PHB-positive transgenic A. thaliana plants. Transgenic plants S8-1-2A and S8-1-2C were cross-pollinated with transgenic plants RedB-2D or RedB-2A. The resulting F1 seeds were sowed in soil and leaf samples from 2-3 week-old plants were analyzed by TEM (micrograph a to e). Some F1 seeds were also soaked in water for 24 hours, the embryo dissected out of the seed coat and the cotyledons analyzed by TEM (micrograph f). (a) Two adjacent leaf mesophyll cells from RedB-2D X S8-1-2A F1 hybrid showing agglomerations of electron-lucent granules in the nucleus. (b) Higher magnification of the nucleus of the upper right cell shown in micrograph a. (c) Nucleus of a leaf mesophyll cell from a RedB-2D X S8-1-2A F1 hybrid showing an agglomeration of granules. (d) Leaf mesophyll cell from a RedB-2A X S8-1-2A F1 hybrid showing electron-lucent granules in the nucleus (N) and vacuole (V). (e) Leaf mesophyll cell from a Red B-2A X S8-1-2A F1 hybrid showing electron-lucent granules in the cytoplasm. (f) Cotyledon cells from a RedB-2A X S8-1-2C F1 hybrid seed showing granules in the nucleus. Arrows indicate agglomerations of electron-lucent granules. Bar = 1 µm for micrograph a, b, c, d, and f. Bar = 0.25 µm for micrograph e.

SUMMARY OF THE INVENTION

The present invention relates to a transgenic plant material containing foreign DNA leading to the production of a polyhydroxyalkanoate.

The present invention further relates to a transgenic plant material containing foreign DNA encoding a peptide which exhibits 3-ketothiolase activity.

The present invention also relates to a transgenic plant material containing foreign DNA encoding

a peptide which exhibits acetoacetyl-CoA reductase activity.

The present invention also relates to a transgenic plant material containing foreign DNA encoding a peptide which exhibits foreign PHB synthase activity.

The present invention relates to a method for introducing bacterial DNA encoding proteins required for the synthesis of a polyhydroxyalkanoate into a plant, which comprises mating by sexual fertilization two plants, which do not produce PHB, each containing foreign DNA encoding one or more different enzymes in a pathway leading to polymerization of hydroxyalkyl-CoA by polyhydroxyalkanoate synthase to produce a plant encoding the polyhydroxyalkanoate.

Thus, the present invention provides a method for producing genetically modified higher plants which produce and accumulate PHB or related polyhydroxyalkanoates. In one embodiment, PHB-producing plants are obtained by stably introducing bacterial genes which encode the enzymes acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase) and poly(3-hydroxybutyrate) synthase into the plants by Ti-plasmid mediated transformation. Because bacterial genes are not normally transcribed in plant cells, the genes are modified so that they are under transcriptional control of a DNA sequence (i.e., a "promoter") which induces trancription in plant cells. The genes are also modified by the addition of an appropriate DNA sequence to the non-coding 3'-region of the genes so that the transcripts produced in plant cells are appropriately polyadenylated.

In one embodiment of the invention, PHB-producing plants are obtained by sexual crosses between two parental lines which do not produce PHB. This is accomplished by cross-pollinating a transgenic plant line homozygous for ectopic copies of a modified PHB synthase gene with a transgenic plant line homozygous for ectopic copies of a modified acetoacetyl-CoA reductase gene. In

this context, the term "ectopic genes" refers to genes which are not normally present in an organism but have been stably integrated into the genome by genetic transformation. To be homozygous for ectopic copies means that, in a diploid organism, both homologous chromosomes have the ectopic gene integrated at the same location within the chromosome.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it is helpful to set forth definitions of certain terms to be used hereinafter.

Transformation means the process for changing the genotype of a recipient organism by the stable introduction of DNA by whatever means.

A transgenic plant is a plant which contains DNA sequences which are not normally present in the species, but were introduced by transformation.

Transcription means the formation of an RNA chain in accordance with the genetic information contained in the DNA.

Translation means the process whereby the genetic information in an mRNA molecule directs the order of specific amino acids during protein synthesis.

A promoter is a DNA fragment which causes transcription of genetic material. For the purposes described herein, promoter is used to denote DNA fragments that permit transcription in plant cells. The CaMV 35S-promoter is a DNA fragment from the cauliflower mosaic virus that causes relatively high levels of transcription in many different tissues of many species of higher plants (Benfey, P. N. and Chua, N. H. Science 250:959-966 (1990)).

A poly-A addition site is a nucleotide sequence which causes certain enzymes to cleave mRNA at a specific site and to add a sequence of adenylic acid residues to the 3'-end of the mRNA.

phbC, phbA, phbB are the gene symbols given to the A. eutrophus genes for PHB synthase, 3-ketothiolase and

acetoacetyl-CoA reductase, respectively (Peoples, O. P. and Sinskey, A. J., J. Biol. Chem. 264:15298-15303 (1989)).

In describing the progeny of transgenic plants, it is useful to adopt a convention which designates how many generations of self-pollination have elapsed since the introduction of DNA. Herein, we designate the original transformant the T0 generation. The progeny resulting from self-pollination of this generation is designated the T1 generation and so on.

In the case of cross-pollination between two distinct parental plants, the resulting progeny from the initial cross-pollination event is designated the F1 generation.

Although the experiments discussed hereinafter concern the plant species Arabidopsis thaliana (L.) Heynhold, the process described is generally applicable to any higher plant for which a method of transformation is available. Similarly, although the process described herein concerns the use of genes from A. eutrophus, the process described is generally applicable to the use of genes from any organism which is capable of synthesis of PHB. It is also clear that, although the process described concerns the production of PHB, the procedure is generally applicable to the production of any polyhydroxyalkanoate which is normally produced in microorganisms by the activity of polyhydroxyalkanoate (PHA) synthase (which includes PHB synthase), and for which the appropriate hydroxyalkyl-CoA substrate is produced in the particular plant.

EXPERIMENTAL DETAILS

Experimental Design

The production of PHB in progeny of transformed plants requires the completion of a sequence of steps as follows: (1) the construction of a series of bacterial plasmids containing promoter fusions, (2) the transfer of these plasmids into Agrobacterium tumefaciens, (3) the use of A. tumefaciens to introduce the genes into cells of the plant (i.e., A. thaliana in this example), (4) the

regeneration of transgenic plants (5) the selection of plants which are homozygous for the ectopic genes (6) analysis of the function of the ectopic genes in the transformed plants to ensure that they are expressed and that the gene products are functional (7) the production of hybrid plants containing two or more different ectopic genes by sexual crosses, (8) the analysis of the hybrid material for the presence of PHB. These steps are described in detail in the following sections.

Construction of Transcriptional Fusions

In order to obtain transcription of the bacterial genes in higher plants, the bacterial genes must be modified by the addition of a plant promoter so that they are transcribed when introduced into higher plants. In addition, it is common practice to add a poly-A addition site to the 3'region of bacterial genes in order to obtain proper expression of the genes in higher plants. Both of these requirements were satisfied by cloning the phbC, phbA and phbB genes from plasmid pTZ18U-PHB into the binary Ti plasmid vector pBI121 (Clonetech, CA). The nucleotide sequence of the phbC, phbA and phbB genes contained within the plasmid pTZ18U-PHB is shown in Figure 2. The relevant restriction enzyme sites used for cloning are indicated as well as the deduced open reading frame for the three genes.

A CaMV 35S-phbA gene fusion was constructed by digesting the plasmid pTZ18U-PHB with restriction enzymes PstI and DdeI. The 1.3 kb restriction fragment containing the coding sequence of the 3-ketothiolase gene was separated from other DNA fragments by agarose gel electrophoresis. The DNA fragment was recovered from the agarose using a DEAE cellulose membrane (Schleicher & Schuell NA-45 DEAE membrane). The staggered ends of the DNA fragment were filled-in by incubating the purified restriction fragment with T4 DNA polymerase and deoxynucleotide triphosphates. The blunt fragment was then cloned into the SmaI site in plasmid pUC18 to produce the plasmid pUC-THIO. The 1.3 kb restriction fragment was

excised from pUC-THIO plasmid by digestion with BamHI and SacI, purified by electrophoresis using a DEAE cellulose membrane and ligated into plasmid pBI121 which had been previously digested with the same two enzymes. The resulting plasmid, designated pBI-THIO, was found to have the A. eutrophus 3-ketothiolase gene in the correct orientation, relative to the CaMV 35S promoter and poly-A addition site of pBI121, so that it would be expected to be expressed in higher plants. A schematic summary of the steps involved in construction of pBI-THIO is presented as Figure 3.

A CaMV 35S-phbC gene fusion was constructed by digesting the plasmid pTZ18U-PHB with restriction enzymes BstBI and Tth111I. The 1.9 kb restriction fragment containing the coding sequence of the PHB synthase gene was separated from other DNA fragments by agarose gel electrophoresis. The DNA fragment was recovered from the agarose using a DEAE cellulose membrane. The staggered ends of the DNA fragment were filled in by incubating the purified restriction fragment with T4 DNA polymerase and deoxynucleotide triphosphates. The blunt fragment was then cloned into the SmaI site in plasmid pUC18 to produce plasmid pUC-SYN. The 1.9 kb restriction fragment was excised from pUC-SYN by complete digestion with BamHI and partial digestion with SacI, purified by electrophoresis using DEAE cellulose membranes and ligated into plasmid pBI121 which had been previously digested with the same two enzymes. The resulting plasmid, designated pBI-SYN, was found to have the A. eutrophus PHB synthase gene in the correct orientation, relative to the CaMV 35S promoter and poly-A addition site of pBI121, so that it would be expected to be expressed in higher plants. A schematic summary of the steps involved in construction of pBI-SYN is presented in Figure 4.

A CaMV 35S-phbB gene fusion was constructed by using a pair of synthetic oligonucleotides for primers in a polymerase chain reaction (PCR) to amplify the phbB gene

from plasmid pTZ18U-PHB. The sequence of the oligonucleotide primers is presented in Figure 5 where they are designated PCR primer #1 and PCR primer #2. The oligonucleotides were designed in such a way that the amplified DNA sequence contained a synthetic BamHI site near the 5'-end of the coding sequence and a synthetic KpnI site at the 3'-end of the sequence. The 790 base pair product of the polymerase chain reaction was separated and purified from agarose gel, restricted with BamHI and KpnI and ligated into plasmid pUC18, which was previously restricted with the same two enzymes, to produce plasmid pUC-RED. The restriction fragment was excised from pUC-RED by digestion with BamHI and SacI, purified by electrophoresis using a DEAE cellulose membrane and ligated into plasmid pBI121 which had been previously digested with the same two enzymes. The resulting plasmid, designated pBI-RED, was found to have the A. eutrophus acetoacetyl-CoA reductase gene in the correct orientation, relative to the CaMV 35S promoter and poly-A addition site of pBI121, so that it would be expected to be expressed in higher plants. A schematic summary of the steps involved in construction of pBI-RED is presented as Figure 5.

The plasmids pBI-SYN, pBI-THIO and pBI-RED were transferred into Agrobacterium tumefaciens strain C58 pGV3850 by electroporation. Plasmid containing colonies were recovered by selection for expression of the kanamycin resistance gene present on the parental plasmid pBI121.

Production of Transgenic Plants

Cells of A. thaliana were transformed by incubating sterile root tissue with cultures of A. tumefaciens carrying the recombinant binary Ti plasmids described in the previous section. Roots from sterile seedlings of A. thaliana race Rschew were transformed as described by Valvekens, D. et al., Proc. Natl. Acad. Sci. USA 85:5536-5540 (1988). Each of the three strains of A. tumefaciens carrying one of the modified phb genes was used to infect A. thaliana root pieces. This resulted in the

recovery of approximately 50 kanamycin resistant callus tissues in each case. Of these, 10-25% gave rise to fertile shoots which produced seeds. Each plant which produced seeds was assigned a different number to indicate that it represented a distinct transformation event.

A total of 11 putative transgenic plants were recovered from tissues treated with A. tumefaciens carrying the plasmid pBI-RED. These designated RedB-2A, -2B, -2C, -2D, -2E, -2F, -2G, -2H, -3A, -5A and RedD-3A. All these transgenic plant lines, except RedB-2F, -2H and -5A, were analyzed in detail as described in the following sections.

A total of 5 putative transgenic plants were recovered from tissues treated with A. tumefaciens carrying the plasmid pBI-THIO. These were designated T3-2A, T4-2A, T4-3A, T4-3B and T4-3C. All these transgenic plant lines, except T4-3C, were analyzed in detail as described in the following sections.

A total of 4 putative transgenic plants were recovered from tissue treated with A. tumefaciens carrying the plasmid pBI-SYN. These were designated S8-1-2A, S8-1-2C, S12-3A and S8PUC-2A. All these trangenic plant lines were analyzed in detail as described in the following sections.

The presence of T-DNA in the putative transgenic plants was verified by sowing seed from the transgenic plants on agar-solidified mineral medium containing 50 µg/ml of kanamycin. This concentration of kanamycin prevents the growth of untransformed A. thaliana plants but permits plants containing the NPTII gene carried on pBI121 or pBI121-derived plasmids to grow normally. The seeds from transgenic plants T4-2A, RedD-3A and S8-1-2A are available from The American Type Culture Collection, Rockville, MD 20852.

Isolation of Putative Homozygous Transgenic Lines

A minimum criterion used to produce homozygous transgenic lines was that all the progeny from an homozygous plant are expected to be kanamycin resistant.

Because the presence of multiple ectopic copies of the NPTII gene from pBI121 at different locations in the genome may cause a similar phenotype, this criterion is most useful when the primary transformation event involves insertion of T-DNA into only one chromosomal location.

In order to identify putative homozygous lines, several kanamycin resistant T1 plants from each transgenic line were grown to maturity in reproductive isolation. The frequency of kanamycin resistance was then determined in samples of approximately 50 T2 seed from each line. If all of the T2 seed from a particular plant were kanamycin resistant, the line was provisionally considered to be homozygous.

Analysis of the Integration of the phb Genes in Transgenic Plants

In order to verify the proper integration of the phb genes in the various trangenic plant lines produced, the genomic DNA of the trangenic plants was analyzed. High molecular weight DNA from control untransformed plants and from T3 transgenic plants transformed with the plasmids pBI-THIO, PBI-RED and pBI-SYN was isolated. The DNAs were digested with the restriction enzymes HindIII, the fragments separated by agarose gel electrophoresis and transferred onto a nylon filter. The restriction enzyme HindIII cuts only once at the 5'end of the CaMV 35S promoter in plasmids pBI-THIO, pBI-RED and pBI-SYN (Figures 3, 4 and 5). Fragments detected using phb gene specific probes should therefore represent junction fragments of the Ti vectors with the plant genomic DNA, or internal fragments of concatamerized Ti vectors. The inserts in plasmids pUC-THIO, pCU-RED and pUC-SYN were excised by treatment with EcoRI and HindIII, purified by agarose gel electrophoresis using DEAE cellulose membranes and labeled with ^{32}p-deoxyribonucleotides by random priming. The labeled phb gene fragments were then used to probe the nylon filters. The filters were hybridized and subsequently washed under high stringency conditions. The

result of these filter hybridizations is shown in Figure 6. None of the three phb genes can be detected in untransformed control plants (Figures 6A, B and C, lane a). The phbA gene was detected in four of the transgenic lines produced by transformation with the plasmid pBI-THIO (Figures 6A, lanes b to e). The phbB gene was detected in seven of the transgenic plants produced by transformation with the plasmid pBI-RED (Figure 6B, lanes f to l). Finally, the phbC gene was detected in three of the transgenic plants produced by transformation with the plasmid pBI-SYN (Figure 6C, lanes m to p). Although the plant line S12-3A was resistant to 50 µg/ml of kanamycin, suggesting the integration of the NPTII gene, no phbC gene could be detected. It is likely that only the fragment of the Ti vector harboring the NPTII gene, and not the phbC gene, was integrated in the genomic DNA of plant line S12-3A.

Analysis of Expression of the phb Genes in Transgenic Plants

In order to determine if the A. eutrophus phb genes were expressed in the various transgenic lines, the cloned genes present in plasmids pUC-THIO, pUC-RED and pUC-SYN were used as probes in filter hybridization experiments. Total RNA was extracted from untransformed control and T3 transgenic plants. The RNA was resolved by electrophoresis in formaldehyde-containing agarose gels and transferred to nylon filters by established procedures. The inserts of plasmids pUC-THIO, pUC-RED and pUC-SYN were excised by treatment with EcoRI and Hind III, purified by electrophoresis using DEAE cellulose membranes and labeled with ^{32}P-deoxyribonucleotides by random priming. The labeled phb genes were used to probe the nylon filters. These experiments showed that none of the three phb probes hybridized to any RNA in the untransformed control plant (Figure 7, lane e, j and r). By contrast, transgenic plants produced by transformation with pBI-THIO had RNA of 1.6 kbp which was complementary to the 3-ketothiolase gene

(Figure 7A, lanes a to d). The CaMV 35S promoter and poly-A addition sequences present on the pBI121-derived plasmids contribute approximately 300 bp to the final length of the mRNAs produced from the phb fusion genes. The level of 3-ketothiolase mRNA was low in plant line T4-3A relative to the other plant lines. Similarly, three of the transgenic lines produced by transformation with pBI-SYN had mRNA of 2.1 kbp corresponding to the PHB synthase gene (Figure 7B, lanes f, g, and i). Transgenic line S12-3A had no detectable mRNA hybridizing to the phbC probe (Figure 7B, lane h). This result is in accordance with the Southern blot analysis showing no integration of the phbC gene in the genomic DNA of line S12-3A (Figure 6C, lane m). Finally, seven transgenic lines produced by transformation with the plamid pBI-RED had mRNA of 1.1 kbp which was complementary to the acetoacetyl-CoA reductase gene (Figure 7C, lanes k to q). Thus, for each of the three phb genes, at least three independent transgenic plants were obtained which expressed complementary RNA of the expected size.

Although the presence of RNA indicates that the genes are transcribed, it does not provide any information that they are translated or that the translation product is functional. This was examined by assaying the transgenic plants for enzyme activity.

Transgenic plants produced by transformation with pBI-THIO were assayed for 3-ketothiolase activity by minor modifications of the assay described by Senior, P. J. and Dawes, E. A., Biochem. J. 134:225-238 (1973). Frozen leaf tissues from T3 plants were homogenized in Tris buffer and the clarified crude extracts were assayed for 3-ketothiolase activity. The results of these experiments are presented in Table 1. Extracts from untransformed A. thaliana plants had very low levels of 3-ketothiolase activity under the assay conditions. By contrast, each of the transgenic plants found to transcribe the phbA gene had substantially increased levels of thiolase activity. This

indicated that the bacterial thiolase gene is functional when expressed in transgenic plants. However, the specific activity of 3-ketothiolase detected in the various transgenic plants was significantly lower compared to extracts prepared from E. coli harboring the phbA gene on the plasmid pTZ18U-PHB.

Table 1. Levels of 3-ketothiolase activity in A. thaliana transgenic plants

Sample	3-Ketothiolase activity[a]
DH5alpha/PHB[b]	9.5
Wild type A. thaliana	0.019
T4-3A transgenic	0.057
T3-2A transgenic	0.42
T4-2A transgenic	0.43
T4-3B transgenic	0.54

[a]Micromoles of acetoacetyl-CoA degraded per minute per milligram of protein. Values are an average of two to four measurements.

[b]E. coli DH5alpha containing the plasmid pTZ18U-PHB harboring the PHB operon.

Transgenic plants produced by transformation with plasmid pBI-RED were assayed for acetoacetyl-CoA reductase activity by minor modifications of the assay described by Senior, P. J. and Dawes, E. A., Biochem. J. 134:225-238 (1973). Leaves from T3 plants were homogenized in potassium phosphate buffer and the clarified extracts were assayed for acetoacetyl-CoA reductase activity. The results of these experiments are presented in Table 2. Extracts from untransformed A. thaliana plants had undetectable levels of acetoacetyl-CoA reductase activity under the assay conditions. By contrast, each of the transgenic plants found to transcribe the phbB gene had high levels of acetoacetyl-CoA reductase activity. This indicates that the bacterial acetoacetyl-CoA reductase gene

is functional when expressed in transgenic plants. Furthermore, the specific activity of acetoacetyl-CoA reductase detected in six of the seven transgenic plants analyzed was significantly higher than in extracts from E. coli harboring the phbB genes on the plasmid pTZ18U-PHB.

Table 2. Levels of acetoacetyl-CoA reductase activity in A. thaliana transgenic plants

Sample	Acetoacetyl-CoA reductase activity[a]
DH5alpha/PHB[b]	1.4
Wild type A. thaliana	<0.03
RedB-2A transgenic	12.5
RedB-2B transgenic	16.2
RedB-2C transgenic	9.1
RedB-2D transgenic	8.8
RedB-2E transgenic	1.6
RedB-2G transgenic	5.2
RedD-3A transgenic	2.3

[a]Micromoles of NADPH reduced per minute per milligram of protein. Values are an average of two to four measurements.

[b]E. coli DH5alpha containing the plasmid pTZ18U-PHB harboring the PHB operon.

Transgenic plants obtained by transformation with the plasmid pBI-SYN were not assayed for the presence of PHB synthase activity because of technical difficulties in measuring the activity of this enzyme in the absence of thiolase and reductase activities.

Production and Analysis of Hybrid Plants

Because higher plants contain an endogenous cytoplasmic 3-ketothiolase activity, the only additional enzymes required to produce PHB are acetoacetyl-CoA reductase and PHB synthase. These two genes were introduced into the same plant by cross-pollinating a transgenic line which was judged to be homozygous for the

acetoacetyl-CoA reductase gene with a transgenic line which was judged to be homozygous for the PHB synthase gene. The hybrid seeds resulting from these crosses were grown in soil for two to three weeks before assaying for the presence of PHB.

In order to determine if the presence in plants of the acetoacetyl-CoA reductase and PHB synthase genes was sufficient for production and accumulation of PHB, extracts of chloroform-soluble material were made from control plants and hybrid plants containing both of these genes. The presence of PHB within these extracts was analyzed by gas chromatography (GC). Two methods were used to prepare plant extracts for GC analysis. These methods exploit both the highly polymerized nature of PHB (10^6 daltons on average for PHB produced from A. eutrophus) and its selective solubility in chlorinated hydrocarbons such as chloroform. Briefly, in method #1, whole leaves are placed in a 1:1 solution of chloroform and water and shaken by inversion for 16 hours at 65°C. Because molecules larger than approximately 50,000 daltons cannot pass through the plant cell wall, only low molecular weight water or chloroform-soluble products are extracted from the leaves under these conditions. The putative high molecular weight PHB is then extracted from the leaves by homogenizing the remaining tissue to disrupt the cell wall, and re-extracting it in a solution of 1:1 chloroform and water for 12 hours at 65°C. In method #2, whole leaf samples are successively extracted for 2 hours at 55°C in 50% ethanol, 2 hours at 55°C in 100% methanol and 15 minutes at 20°C in 100% diethyl ether. The remaining tissue is then homogenized and extracted in chloroform at 100°C for 4 hours. The products present in the final chloroform extract obtained from both of these methods were transesterified with ethanol and hydrochloric acid and analyzed by gas chromatography. The retention time of the transesterified plant products were compared to

transesterified commercial PHB purified from A. eutrophus (Sigma Chemical Co., MO).

Transgenic plants S8-1-2A and/or S8-1-2C were cross-pollinated with transgenic plants RedB-2A, -2C, -2D, -2G and RedD-3A. The resulting F1 seeds were sowed in soil and leaf samples or whole shoots of 2-3 week-old plants were collected and analyzed for the presence of PHB. An example of the results obtained using purification method #1 are shown in Figure 8. A product present in the extracts of F1 plants having both the acetoacetyl-CoA reductase and the PHB synthase transgenes has a retention time identical to ethyl-hydroxybutyrate, as determined by comparison with the retention time of the transesterified product of commercial PHB. This new product, tentatively identified as ethyl-hydroxybutyrate, was only detected in F1 hybrid plants having both an active acetoacetyl-CoA reductase transgene and a PHB synthase transgene. A similar product could not be detected in transgenic plants having only one of the above mentioned genes or in untransformed A. thaliana plants. Furthermore, this product could not be detected in chloroform extracts of plant tissues which had not been previously homogenized. This indicates that the ethyl-hydroxybutyrate is derived from a high molecular weight precursor.

The identity of the new plant product having a retention time identical to ethyl-hydroxybutyrate was analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed by the MSU-NIH Mass Spectrometry Facility. Figure 9A shows the mass spectrometric spectra of ethyl-hdydroxybutyrate prepared from authentic PHB. Figure 9B shows the mass spectrum of the putative ethyl-hydroxybutyrate extracted from an F1 hybrid plant which resulted from a cross between transgenic plants S8-1-2A and RedB-2C. The results indicated that the new plant product eluting with the same retention time as ethyl-hdyroxybutyrate also has the same fragmentation pattern as an authentic sample of ethyl-hyroxybutyrate.

The fact that this new product can only be detected in extracts from leaf tissue which has previously been homogenized indicates that the ethyl-hydroxybutyrate is derived from material having a molecular weight greater than approximately 50,000 daltons (the approximate porosity of plant cell walls). Together, these data indicate that transgenic plants containing both the acetoacetyl-CoA reductase and the PHB synthase genes accumulate polyhydroxybutyrate. Table 3 shows a summary of the F1 plants that were analyzed by GC and GC-MS. Based on the GC analysis, the amount of PHB accumulated in leaves of F1 hybrids ranged from approximately 5 µg of PHB per gram of fresh weight of leaves for F1 hybrids between RedD-3A and S8-1-2C, to approximately 100 µg of PHB per gram fresh weight of leaves from F1 hybrid between RedB-2C and S8-1-2A.

Table 3. Summary of evidence for production of PHB in F1 hybrid plants

Parental Transgenic Line[a]	Parental Transgenic Line[a]	
	S8-1-2C	S8-1-2A
RedD-3A	GC[b] MS[c]	TEM[d] (leaf)
RedB-2A	TEM (seed)	GC TEM(leaf, seed)
RedB-2G		GC MS TEM(leaf)
RedB-2C	TEM (leaf)	GC MS
RedB-2D		TEM (leaf)

[a]Transgenic lines harboring the acetoacetyl-CoA reductase transgene were cross-pollinated with transgenic lines harboring the PHB synthase. The resulting F1 hybrids were analyzed for production of PHB.

[b]Evidence for production of PHB by gas chromatography analysis

[c]Evidence for production of PHB by gas chromatography-mass spectrometry.

[d]Detection of electron-lucent granules by transmission electron microscopy. In parenthesis is indicated the plant tissue analyzed.

[e]All blank spaces indicate that the analysis has not been performed.

Visual Inspection of PHB Granules in Hybrid Plants

Transmission electron microscopy (TEM) of bacteria accumulating PHB revealed electron-lucent granules of 0.2 to 0.5 μm in diameter surrounded by a membrane coat of about 2 nm thick (Lundgren, D. G., Pfister, R. M. and

Merrick, J. M., J. Gen. Microbiol. 34:441-446 (1964)). To determine if similar granules could be detected in hybrid plants shown to be positive for PHB production by GC-MS analysis, plant tissues were examined by transmission electron microscopy. Transgenic plants S8-1-2A and/or S8-1-2C were cross-pollinated to transgenic RedB-2A, -2C, -2D, -2G and RedD-3A. The resulting F1 hybrid seeds and mature leaf material were fixed for analysis by transmission electron microscopy. Briefly, tissues were fixed in 3% glutaraldehyde and 1% osmium tetroxide and embedded in epoxy resin. Sections of 80-90 nm were stained with 5% uranyl acetate and lead citrate.

In one series of experiments, the F1 seeds were sowed in soil and leaves from 2-3 week-old plants were collected for TEM analysis. Inspection of the cells present in the leaves revealed the presence of agglomerations of electron-lucent granules. These granules were detected in all analyzed F1 hybrid plants resulting from crosses between transgenics having the PHB synthase gene and transgenics having the acetoacetyl-CoA reductase gene. Examples are shown in Figure 10. Similar granules were never detected in the parental transgenic lines having only the PHB synthase or the acetoacetyl-CoA reductase genes, nor was it detected in untransformed A. thaliana. In F1 hybrid leaf tissues, the granules were detected in mesophyll cells (Figure 10 micrograph a to e). The agglomerate of electron-lucent granules were detected most frequently in the nucleus (Figure 10, micrograph a to c), but similar structures were also detected in the cytoplasm (Figure 10, micrograph e) and the vacuole (Figure 10, micrograph d) of the F1 hybrid leaf tissues. In the nucleus and cytoplasm, individual granules could reach a maximum size of approximately 0.18 µm. In the vacuoles, the granules were generally larger, reaching a maximum diameter of approximately 0.55 µm. At higher magnification, the nuclear granules appear to be surrounded by electron-dense material. Both the size and apparent

structure of these granules are very similar to granules observed in bacteria which accumulate PHB.

In a second series of experiments, F1 seeds were soaked in water for 24 hours, the embryos were dissected out of the seed coat and tissues were fixed. Analysis of the embryonic cotyledons revealed the presence of agglomeration of electron-lucent granules in the nucleus (Figure 10, micrograph f). The granules could reach a maximum diameter of 0.18 μm. These granules could only be detected in the nucleus of F1 hybrid embryos resulting from crosses between transgenics having the PHB synthase gene and transgenics having the acetoacetyl-CoA reductase gene. No granules could be detected in either of the parental transgenic plants having only one of the ectopic genes, or in untransformed wild type A. thaliana. Table 3 shows a summary of the F1 plants that were analyzed by TEM.

The data described above show a positive correlation between detection of PHB by GC-MS and the presence of granules at the electron microscope level. The size, shape and presence of electron-dense material surrounding the individual granules very closely resembles the granules present in bacteria producing PHB. Finally, both the detection of PHB by GC-MS and the presence of electron-lucent granules are only observed in hybrid plants possessing both the acetoacetyl-CoA reductase and the PHB synthase transgenes. Together, these data indicate that the granules observed in F1 hybrid plants are composed of polyhydroxybutyrate.

DISCUSSION

In these studies, it has been demonstrated that bacterial genes encoding enzymes required for PHB synthesis can be stably introduced into a higher plant in such a way that the genes are transcribed and produce transcripts of the expected size. It was further shown that, in the case of the phbA and phbB genes, the presence of these genes in transgenic plants confers an increase in the level of 3-ketothiolase or acetoacetyl-CoA reductase enzyme

activity, respectively. Thus, it is clear that these two gene products are functional when translated in the plant. Because of technical difficulties associated with assaying PHB synthase activity directly, the amount of PHB synthase activity in the transgenic plants was not determined.

It was shown that only plant extracts from F1 transgenic plants expressing both the acetoacetyl-CoA reductase and PHB synthase produce a new high molecular weight chloroform-soluble compound, which upon transesterification with ethanol and hydrochloric acid, produces ethyl-hydroxybutyrate. These data indicate that the new compound is polyhydroxybutyrate. In addition, these data are an indirect evidence for the production of a functional PHB synthase in transgenic plants. This is important since an in vitro assay for the PHB synthase activity could not be performed. Furthermore, production of PHB also indirectly indicate that D(-)-hyroxybutyryl-CoA, the substrate for the PHB synthase, is produced in plants. This hydroxyacyl-CoA is not naturally found in plants.

Transmission electron microscopy further substantiates the claim that PHB is produced in transgenic plants. Analysis of embryonic cotyledons and mature leaves of F1 transgenic plants expressing both the acetoacetyl-CoA reductase and the PHB synthase revealed agglomerates of electron-lucent granules having a size and structure very similar to granules found in bacteria producing PHB, such as A. eutrophus. These granules were found most frequently in the nucleus, but were also detected in the vacuole and the cytoplasm of F1 hybrid plants.

In the experiments described in this work, the products of the phbA, phbB, and phbC genes from A. eutrophus are most likely expressed in the cytoplasm, since no specific amino acid sequences were added to the proteins to target them specifically into any organelles. Since the cytoplasm of plant cells already contains a 3-ketothiolase, only the additional expression of the acetoactyl-CoA

reductase and PHB synthase was required to produce PHB. The fact that granules are found in the nucleus and vacuoles is not necessarily contradictory with the expression of the enzymes in the cytoplasm. Since nuclear membranes dissemble and reassemble during cell division, PHB granules initially produced in the cytoplasm could be entrapped within the newly reforming membranes of the nucleus. Alternatively, because of their hydrophobicity, PHB granules could pass through the membranes of the nucleus or vacuole.

In an alternative approach, PHB production could be localized to a specific plant cell organelle through targeted expression of the enzymes involved in PHB synthesis to the organelle. In this case, if the targeted organelle does not express an active 3-ketothiolase, expression of an exogenous 3-ketothiolase activity would be required, in addition to the acetoacetyl-CoA reductase and the PHB synthase, for the production of PHB.

The long term goal of PHB or PHA production in higher plants is to divert carbon away from major storage compounds such as lipid, starch or terpenoids, to channel it towards PHA synthesis. This goal will require tissue-specific expression as well as potentially organelle-specific expression of the enzymes involved in PHA synthesis.

Oil producing crops are likely targets for genetic engineering. Lipids are synthesized in the plastid using acetyl-CoA, the same precursor used in synthesis of PHB and other PHA. Therefore, genetic engineering of oil crops will require targeting the PHA biosynthetic enzymes into the plastid. Examples of oil crops that could be engineered for PHA production are rapeseed, sunflower and oil palm. Rapeseed and sunflower are major crops in North America and can be transformed with foreign DNA. Alternatively, PHA production could be targeted into the mesocarp of the oil palm fruit. Because lipids produced in the mesocarp are not essential for the survival of the

tree or the embryo, the production of PHA should have no deleterious effects on palm trees. Unfortunately, no transformation techniques are yet available for oil palm.

PHA production could also be targeted to the roots and tubers of sugar beets and potatoes, crops which accumulate large amounts of starch. The major problem with this approach is that since starch and PHA do not use the same precursors, potentially multiple modifications in carbon metabolism will be required before carbon could be diverted away from starch into PHA.

Possibly the most direct approach to PHA production would be to use crops accumulating large amounts of terpenoids, such as carrot which accumulates carotenoids, or the mexican yam which accumulates sterols. Since terpenoids use the same precursors as PHA (acetyl-CoA and acetoacetyl-CoA), diverting carbon into PHA production could be more easily achieved.

MATERIALS AND METHODS

Construction of DNA Recombinants

E. coli strain DH5alpha harboring plasmids were grown in LB broth supplemented with kanamycin (50 µg/ml) or ampicillin (50 µg/ml). Large-scale preparations of plasmid DNA was done by the alkaline lysis and polyethylene glycol precipitation procedure as described by Sambrook, J., Fritsch E. F. and Maniatis, T., Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989). Plasmid DNA was cleaved with restriction endonucleases according to the manufacturer's recommendations (New England Biolabs, Mass; Promega Corp., WI; Boehringer Mannheim Biochemicals, IN; Stratagene, CA), separated by agarose gel electrophoresis and visualized by ethidium bromide staining as described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989). The DNA fragments were recovered from the agarose gel with DEAE membranes (NA-45 DEAE membrane, Schleicher and Schuell, Inc., NH). Briefly, DNA is electrophoresed

onto a strip of NA-45 and the membrane is washed in 0.15 M NaCl, 0.1 mM EDTA and 20 mM Tris-HCl (pH 8). The DNA is then eluted in 1.0 M NaCl, 0.1 mM EDTA and 20 mM Tris-HCl (pH 8) at 65°C for 1 to 2 hours. The DNA is further purified by phenol-chloroform extraction and ethanol precipitation. In some experiments, the recessed 3' termini of DNA fragments were converted into blunt ends with T4 DNA polymerase using the protocol described in Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989). Ligation of DNA fragments with cohesive or blunt ends was done at 14°C for 16 hours in buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5% (w/v) polyethylene glycol 8000, 0.5 mM ATP and 5 mM dithiothreitol as described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989). A fraction of the ligation reaction was transferred into E. coli by the rubidum chloride method as described by Hanahan, D., J. Mol. Biol. 166:557-580 (1983). The transformed bacteria were plated on agar plates containing LB broth and either 50 µg/ml kanamycin or 50 µg/ml ampicillin. Bacterial colonies containing recombinant plasmids were identified by hybridization with ^{32}P-labeled DNA probes as described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989), except that nylon membranes (Hybond-N, Amersham, IL) were used instead of nitrocellulose membranes. Preparation of radiolabeled DNA probes and hybridization are described in a following section. Small-scale preparation of plasmid DNA was done by the alkaline lysis method as described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989).

Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer according to the manufacturer's instructions (Applied Biosystems, CA). The

oligonucleotides with a dimethoxytrityl group attached to the 5' ends were purified on a Varian 5000 HPLC equipped with a C_{18} column (Varian Instrument Group, TX). The oligonucleotides were resuspended in 0.1M triethylamine and injected onto a C_{18} column preequilibrated with 12% acetonitrile/88% 0.1 M triethylamine-acetate (pH7) (solvent A). The HPLC program was set as follows: flow rate, 0.9 ml/min; maximum pressure, 200 psi; time 0 min, 88% solvent A/12% solvent B (acetonitrile); time 3 min, 88% solvent A/12% solvent B; time 21 min, 65% solvent A/35% solvent B; time 25 min, 65% solvent A/35% solvent B. The purified oligonucleotides were detritylated in 80% acetic acid for 10 min and dried under nitrogen. The oligonucleotides were dissolved in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA, extracted three times with equal volumes of ethyl acetate and precipitated with ethanol.

Polymerase chain reaction (PCR) was performed using a Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer, CT). The reaction mixture contained 100 pmoles of oligonucleotides, PCT primer #1 and #2 (see Fig. 5), 200 ng of plasmid pTZ18U-PHB linearized with the restriction enzyme EcoRI, 125 µM dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin and 2.5 units of Taq polymerase (Perkin-Elmer, CT). The DNA thermal cycler program was as follows: 3 min at 94°C, 40 cycles of the sequence 1 min at 94°C - 3 min at 55°C - 3 min at 72°C, and finally 7 min at 72°C. The PCR product was isolated by agarose gel electrophoresis and elution with DEAE cellulose membrane.

Production of Transgenic Plants

The Ti plasmid vectors used to produce transgenic plants were first transferred into Agrobacterium tumefaciens strain C58-pGV3850 by electroporation (Zabrisky, P. et al., EMBO 2:2143-2150 (1983); and Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989)). Arabidopsis thaliana race Rschew were grown

aseptically on vertical petri plates containing mineral elements, 0.8% agar (Difco) and 1% sucrose as described by Estelle, M. A. and Somerville, C., Mol. Gen. Genet. 206:200-206 (1987) and Schiefelbein, J. W. and Somerville, C. R., Plant Cell 2:235-243 (1990). Roots from 10 to 12 day-old plants were excised and used for transformation as described by Valvekens, D., Van Montagu, M. and Van Lijsebettens, M., Proc. Natl. Acad. Sci USA 85:5536-5540 (1988).

Seeds from TO and T1 transgenics plants were grown on media containing mineral elements, 1% sucrose, 0.8% agar (Difco) and 50 µg/ml kanamycin. After 10 to 14 days of growth, kanamycin resistant (Km^r) transgenic plants had green leaves while untransformed kanamycin sensitive (Km^s) plants had yellow leaves. At this stage, Km^r plants could be removed from the agar plates and transplanted into fertilized soil.

Extraction and Restriction Endonuclease Cleavage of Genomic DNA

Wild type and transgenic plants were grown in soil for 2 to 3 weeks and approximately 5 g of leaf material was collected and frozen in liquid nitrogen. High molecular weight DNA was extracted from the frozen plant tissues as described by Rogers, S. C. and Bendich, A. J., Plant Molecular Biology Manual A6:1-10 (1988). Restriction endonuclease cleavage with the enzyme HindIII was performed under the conditions recommended by the manufacturer (New England Biolabs Inc., Mass).

Agarose Gel Electrophoresis and Hybridization Procedure

DNA analysis by agarose gel electrophoresis and transfer to nylon membranes (Hybond-N, Amersham, Il) were done using established procedures described by Southern, E. M., J. Mol. Biol. 38:503-517 (1975) and Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989). Specific cloned DNA fragments to be used as probes were excised from the vector with appropriate restriction

endonucleases, the inserts were purified from the vector by agarose gel electrophoresis and electroelution using DEAE cellulose membranes. Probes were labeled with ^{32}P-deoxyribonucleotides by the random primer extension method using hexamers as described by Feinberg, A. P. and Volgelstein, B., Anal. Biochem. 136:6-13 (1983). Nylon filters were hybridized with labeled probes and exposed on film as described by Poirier, Y. and Jolicoeur, P., J. Virol. 63:2088-2098 (1989).

RNA Isolation and Electrophoresis

Total RNA was isolated from frozen leaf samples as described by Puissant, C. and Houdebine, L. M., BioTechniques 8:148-149 (1990). The isolated RNA was separated by electrophoresis in agarose gel containing formaldehyde and transferred onto nylon membranes (Hybond-N, Amersham, Il) as described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press (1989). The nylon filters were hybridized with labeled probes as described in the previous section.

Assay for 3-Ketothiolase Activity

One gram of frozen leaf samples were homogenized in 2 ml of ice-cold buffer containing 100 mM Tris-HCl (pH 8.0), 40 mM $MgCl_2$ and 5 mM beta-mercaptoethanol. The homogenate was clarified by centrifugation at 10000X g for 2 min and the supernatant transferred to a fresh tube. The protein content of the extract was measured by the Bradford assay using the BioRad protein assay kit (BioRad Laboratories, CA). Between 3 to 30 µg of plant protein extracts were used per assay. Protein extracts were also prepared from bacteria. In this case, stationary cultures of bacteria were pelleted by centrifugation, washed once with ice-cold assay buffer and resuspended in 200 µl of the same buffer. The bacterial suspension was lysed by sonication, the homogenate clarified by centrifugation and the protein content of the extract determined by the Bradford assay. Between 0.2 to 1 µg of bacterial protein

extract was used per assay. Activity of the 3-ketothiolase enzyme in the different extracts was assayed according to the procedure of Senior, P. J. and Dawes, E. A., Biochem. J. 134:225-238 (1973).

Assay for Acetoacetyl-CoA Reductase Activity

One gram of frozen leaf samples were homogenized in 2 ml of ice-cold buffer containing 100 mM KH_2PO_4 (pH 5.5), 0.02 mM $MgCl_2$ and 4.0 mM beta-mercaptoethanol. The homogenate was clarified by centrifugation at 10000X g for 2 min and the supernatant transferred to a fresh tube. The protein content of the extract was measured by the Bradford assay using the BioRad protein assay kit. Between 0.8 to 10 µg of plant protein extract was used per assay. Bacterial extracts were also prepared in the assay buffer essentially as described in the previous section. Activity of the acetoacetyl-CoA reductase enzyme was assayed according to the procedure of Senior, P. J. and Dawes, E. A., Biochem. J. 134:225-238 (1973).

Gas Chromatography and Mass Spectroscopy Analysis

Two methods were used to prepare plant extracts for GC analysis. In method #1, between 0.005 and 0.05 g of fresh or frozen plant material (leaves or whole shoots) was extracted in 1 to 2 ml of a 1:1 solution of chloroform and water at 65°C for 16 hours with constant agitation. The plant material was then homogenized in water and re-extracted in a 1:1 solution of chloroform and water for 16 hours at 65°C with constant agitation. The chloroform phase was transferred to a new tube and extracted once with an equal volume of water. The final volume of the chloroform phase was adjusted to 0.5 ml and used for transesterification with ethanol and HCl as described below. In method #2, between 0.005 to 0.15 g of frozen or fresh plant material was successively extracted in 50% ethanol at 55°C for 2 hours, 100% methanol at 55°C for 2 hours and 100% diethylether for 15 minutes at room temperature. The remaining tissue was then homogenized in water, dried and extracted in 0.5 ml of chloroform for 4 hours at 100°C.

The final chloroform extracts (0.5 ml) obtained by method #1 and #2 was transesterified by adding 0.2 ml of concentrated HCl and 1.7 ml of 100% ethanol and heating at 100°C for 2 hours. The reaction mixture was then cooled down to room temperature, the chloroform phase extracted twice with 2 ml of 0.9% NaCl (w/v) and the final organic phase reduced to 100 µl. As a standard, commercial PHB (Sigma Chemical Co., MO) was dissolved in warm chloroform and 1 mg was transesterified as described above.

The chloroform phase containing the ethyl esters were transferred to a GC vial for injection of 1 µl into a Hewlett Packard 5890 series II GC equipped with a programmable autosampler and a SP-2330 glass capillary column (Supelco, PA). The approximate linear velocity was 20 cm/s with helium as the carrier gas. The temperature of the injection port was set at 220°C, and that of the flame ionization detector port was set at 220°C. The following temperature profile was used: 4 minutes at 65°C, followed by a temperature increase rate of 20°C/minute up to 195°C, 3.5 minutes at 195°C, a post-run temperature decrease rate of 20°C/minute down to 65°C.

The identity of peaks of interest was established by GC-mass spectrometry. Electron impact mass spectral data was obtained on a JEOL JMS-AX505H mass spectrometer coupled with a Hewlett Packard 5890 GC. The following parameters were used: source temperature, 200°C; ionization current, 100 µA; accelerating voltage, 3 keV. A J & W Scientific Co. column DB-225 was directly inserted into the mass spectrometer source and helium was used as carrier. The splitless injector was held at 260°C and the transfer line at 260°C. The same GC oven temperature profile was used (see previous paragraph).

Transmission Electron Microscopy

Plant tissues were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1.5-2 hours at room temperature. The samples were washed 4 times in 0.1 M phosphate buffer (pH 7.2) and fixed in 1% OsO_4 in phosphate

buffer for 2 hours at room temperature. The tissues were then dehydrated in a graded ethanol series and embedded in Spurrs epoxy resin. Sections of 80-90 nm were cut, placed on a copper grid and stained in 5% uranyl acetate for 30 to 45 minutes, followed by staining in Reynolds lead citrate for 3 to 4 minutes. Sections were viewed in a JEOL 100CXII transmission electron microscope operated at 80 kV.

Other Plants

Although the specific example of the invention described here involved the plant Arabidopsis thaliana and genes from Alcaligenes eutrophus, the invention is of general utility. The claims pertaining to production of polyhydroxybutyrate and/or polyhydroxyalkanoate in plants is not limited to Arabidopsis thaliana, or linked specifically to the use of genes from Alcaligenes eutrophus. The claims described below describe a general method for the production of polyhydroxyalkanoate in plants through the introduction of foreign DNA material into plant cells. Such plants include the plants discussed previously and carrot, sunflower, tobacco, tomato and potato, for instance.

The seeds from the various lines of plants have been deposited under the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. These lines include RedD-3A (ATCC 75044) containing the acetoacetyl-CoA reductase gene; S8-1-2A (ATCC 75043) containing the PHB synthase gene and T4-2A (ATCC 75042) containing the 3-ketothiolase gene. The genes are each shown in Sequence ID NO: 1.

The foregoing specific description is only illustrative of the present invention and it is intended that the present invention be limited only by the hereinafter appended claims.

APPENDIX I

(1) GENERAL INFORMATION:

(i) Applicants: Chris Somerville, Yves Poirier, Douglas Dennis

(ii) Title of Invention: Transgenic Plant Materials Producing Polyhydroxyalkanoates

(iii) Number of Sequences: 1

(iv) Correspondence Address:

(A) Addressee: Ian C. McLeod

(B) Street: 2190 Commons Parkway

(C) City: Okemos

(D) State: Michigan

(E) County: Ingham

(F) Zip Code: 48864

(v) Computer Readable Form:

(A) Medium Type: Diskette 5.25 inch, 360 Kb Storage

(B) Computer: IBM AT

(C) Operating System: MS-DOS (version 4)

(D) Software: Wordperfect 5.1

(viii) Attorney/Agent Information:

(A) Name: Ian C. McLeod

(B) Registration No.: 20,931

(C) Reference/Docket Number: MSU 4.1-131

(ix) Telecommunication Information:

(A) Telephone: (517) 347-4100

(B) Telefax: (517) 347-4103

(2) Information for SEQ ID NO: 1

(i) Sequence Characteristics:

(A) Length: 4980 base pairs

(B) Type: Nucleic Acid Encoded Precursor Peptides

(C) Strandedness: Double

(D) Topology: Linear

(ii) Molecule Type:

(A) Description: Genomic DNA

(iii) HYPOTHETICAL: No.

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

(A) Organism: Alcaligenes eutrophus

(vii) IMMEDIATE SOURCE:

(A) Library: genomic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

```
1     CCCGGGCAAGTACCTTGCCGACATCTATGCGCTGGCGCGCACGCGCCTGGCGCGCGCCGG

61    CTGTACCGAGGTCTACGGCGGCGACGCCTGCACCGTGGCCGACGCCGGTCGCTTCTACTC

121   CTATCGGCGCGATGGCGTGACCGGCCGCATGGCCAGCCTGGTCTGGCTGGCGGACTGAGC

181   CCGCCGCTGCCTCACTCGTCCTTGCCCCTGGCCGCCTGCGCGCGCTCGGCTTCAGCCTTG

241   CGTCGGCGGCGGCCGGGCGTGCCCATGATGTAGAGCACCACGCCACCGGCGCCATGCCAT

301   ACATCAGGAAGGTGGCAACGCCTGCCACCACGTTGTGCTCGGTGATCGCCATCATCAGCG

361   CCACGTAGAGCCAGCCAATGGCCACGATGTACATCAAAAATTCATCCTTCTCGCCTATGC

421   TCTGGGGCCTCGGCAGATGCGAGCGCTGCATACCGTCCGGTAGGTCGGGAAGCGTGCAGT

481   GCCGAGGCGGATTCCCGCATTGACAGCGCGTGCGTTGCAAGGCAACAATGGACTCAAATG

541   TCTCGGAATCGCTGACGATTCCCAGGTTTCTCCGGCAAGCATAGCGCATGGCGTCTCCAT

601   GCGAGAATGTCGCGCTTGCCGGATAAAAGGGGAGCCGCTATCGGAATGGACGCAAGCCAC

661   GGCCGCAGCAGGTGCGGTCGAGGGCTTCCAGCCAGTTCCAGGGCAGATGTGCCGGCAGAC

721   CCTCCCGCTTTGGGGGAGGCGCAAGCCGGGTCCATTCGGATAGCATCTCCCCATGCAAAG

781   TGCCGGCCAGGGCAATGCCCGGAGCCGGTTCGAATAGTGACGGCAGAGAGACAATCAAAT

841   CATGGCGACCGGCAAAGGCGCGGCAGCTTCCACGCAGGAAGGCAAGTCCCAACCATTCAA
S1     MetAlaThrGlyLysGlyAlaAlaAlaSerThrGlnGluGlyLysSerGlnProPheLy

901   GGTCACGCCGGGGCCATTCGATCCAGCCACATGGCTGGAATGGTCCCGCCAGTGGCAGGG
S21   sValThrProGlyProPheAspProAlaThrTrpLeuGluTrpSerArgGlnTrpGlnGl

961   CACTGAAGGCAACGGCCACGCGGCCGCGTCCGGCATTCCGGGCCTGGATGCGCTGGCAGG
S41   yThrGluGlyAsnGlyHisAlaAlaAlaSerGlyIleProGlyLeuAspAlaLeuAlaGl
```

```
1021 CGTCAAGATCGCGCCGGCGCAGCTGGGTGATATCCAGCAGCGCTACATGAAGGACTTCTC
S61   yValLysIleAlaProAlaGlnLeuGlyAspIleGlnGlnArgTyrMetLysAspPheSe

1081 AGCGCTGTGGCAGGCCATGGCCGAGGGCAAGGCCGAGGCCACCGGTCCGCTGCACGACCG
S81   rAlaLeuTrpGlnAlaMetAlaGluGlyLysAlaGluAlaThrGlyProLeuHisAspAr

1141 GCGCTTCGCCGGCGACGCATGGCGCACCAACCTCCCATATCGCTTCGCTGCCGCGTTCTA
S101 gArgPheAlaGlyAspAlaTrpArgThrAsnLeuProTyrArgPheAlaAlaAlaPheTy

1201 CCTGCTCAATGCGCGCGCCTTGACCGAGCTGGCCGATGCCGTCGAGGCCGATGCCAAGAC
S121 rLeuLeuAsnAlaArgAlaLeuThrGluLeuAlaAspAlaValGluAlaAspAlaLysTh

1261 CCGCCAGCGCATCCGCTTCGCGATCTCGCAATGGGTCGATGCGATGTCGCCCGCCAACTT
S141 rArgGlnArgIleArgPheAlaIleSerGlnTrpValAspAlaMetSerProAlaAsnPh

1321 CCTTGCCACCAATCCCGAGGCGCAGCGCCTGCTGATCGAGTCGGGCGGCGAATCGCTGCG
S161 eLeuAlaThrAsnProGluAlaGlnArgLeuLeuIleGluSerGlyGlyGluSerLeuAr

1381 TGCCGGCGTGCGCAACATGATGGAAGACCTGACACGCGGCAAGATCTCGCAGACCGACGA
S181 gAlaGlyValArgAsnMetMetGluAspLeuThrArgGlyLysIleSerGlnThrAspGl

1441 GAGCGCGTTTGAGGTCGGCCGCAATGTCGCGGTGACCGAAGGCGCCGTGGTCTTCGAGAA
S201 uSerAlaPheGluValGlyArgAsnValAlaValThrGluGlyAlaValValPheGluAs

1501 CGAGTACTTCCAGCTGTTGCAGTACAAGCCGCTGACCGACAAGGTGCACGCGCGCCCGCT
S221 nGluTyrPheGlnLeuLeuGlnTyrLysProLeuThrAspLysValHisAlaArgProLe

1561 GCTGATGGTGCCGCCGTGCATCAACAAGTACTACATCCTGGACCTGCAGCCGGAGAGCTC
S241 uLeuMetValProProCysIleAsnLysTyrTyrIleLeuAspLeuGlnProGluSerSe

1621 GCTGGTGCGCCATGTGGTGGAGCAGGGACATACGGTGTTTCTGGTGTCGTGGCGCAATCC
S261 rLeuValArgHisValValGluGlnGlyHisThrValPheLeuValSerTrpArgAsnPr

1681 GGACGCCAGCATGGCCGGCAGCACCTGGGACGACTACATCGAGCACGCGGCCATCCGCGC
S281 oAspAlaSerMetAlaGlySerThrTrpAspAspTyrIleGluHisAlaAlaIleArgAl

1741 CATCGAAGTCGCGCGCGACATCAGCGGCCAGGACAAGATCAACGTGCTCGGCTTCTGCGT
S301 aIleGluValAlaArgAspIleSerGlyGlnAspLysIleAsnValLeuGlyPheCysVa

1801 GGGCGGCACCATTGTCTCGACCGCGCTGGCGGTGCTGGCCGCGCGCGGCGAGCACCCGGC
S321 lGlyGlyThrIleValSerThrAlaLeuAlaValLeuAlaAlaArgGlyGluHisProAl
```

```
1861 CGCCAGCGTCACGCTGCTGACCACGCTGCTGGACTTTGCCGACACGGGCATCCTCGACGT
S341 aAlaSerValThrLeuLeuThrThrLeuLeuAspPheAlaAspThrGlyIleLeuAspVa

1921 CTTTGTCGACGAGGGCCATGTGCAGTTGCGCGAGGCCACGCTGGGCGGCGGCGCCGGCGC
S361 lPheValAspGluGlyHisValGlnLeuArgGluAlaThrLeuGlyGlyGlyAlaGlyAl

1981 GCCGTGCGCGCTGCTGCGCGGCCTTGAGCTGGCCAATACCTTCTCGTTCTTGCGCCCGAA
S381 aProCysAlaLeuLeuArgGlyLeuGluLeuAlaAsnThrPheSerPheLeuArgProAs

2041 CGACCTGGTGTGGAACTACGTGGTCGACAACTACCTGAAGGGCAACACGCCGGTGCCGTT
S401 nAspLeuValTrpAsnTyrValValAspAsnTyrLeuLysGlyAsnThrProValProPh

2101 CGACCTGCTGTTCTGGAACGGCGACGCCACCAACCTGCCGGGGCCGTGGTACTGCTGGTA
S421 eAspLeuLeuPheTrpAsnGlyAspAlaThrAsnLeuProGlyProTrpTyrCysTrpTy

2161 CCTGCGCCACACCTACCTGCAGAACGAGCTCAAGGTACCGGGCAAGCTGACCGTGTGCGG
S441 rLeuArgHisThrTyrLeuGlnAsnGluLeuLysValProGlyLysLeuThrValCysGl

2221 CGTGCCGGTGGACCTGGCCAGCATCGACGTGCCGACCTATATCTACGGCTCGCGCGAAGA
S461 yValProValAspLeuAlaSerIleAspValProThrTyrIleTyrGlySerArgGluAs

2281 CCATATCGTGCCGTGGACCGCGGCCTATGCCTCGACCGCGCTGCTGGCGAACAAGCTGCG
S481 pHisIleValProTrpThrAlaAlaTyrAlaSerThrAlaLeuLeuAlaAsnLysLeuAr

2341 CTTCGTGCTGGGTGCGTCGGGCCATATCGCCGGTGTGATCAACCCGCCGGCCAAGAACAA
S501 gPheValLeuGlyAlaSerGlyHisIleAlaGlyValIleAsnProProAlaLysAsnLy

2401 GCGCAGCCACTGGACTAACGATGCGCTGCCGGAGTCGCCGCAGCAATGGCTGGCCGGCGC
S521 sArgSerHisTrpThrAsnAspAlaLeuProGluSerProGlnGlnTrpLeuAlaGlyAl

2461 CATCGAGCATCACGGCAGCTGGTGGCCGGACTGGACCGCATGGCTGGCCGGGCAGGCCGG
S541 aIleGluHisHisGlySerTrpTrpProAspTrpThrAlaTrpLeuAlaGlyGlnAlaGl

2521 CGCGAAACGCGCCGCGCCCGCCAACTATGGCAATGCGCGCTATCGCGCAATCGAACCCGC
S561 yAlaLysArgAlaAlaProAlaAsnTyrGlyAsnAlaArgTyrArgAlaIleGluProAl

2581 GCCTGGGCGATACGTCAAAGCCAAGGCATGACGCTTGCATGAGTGCCGGCGTGCGTCATG
S581 aProGlyArgTyrValLysAlaLysAla*

2641 CACGGCGCCGGCAGGCCTGCAGGTTCCCTCCCGTTTCCATTGAAAGGACTACACAATGAC
T1                                                          MetTh
```

```
2701 TGACGTTGTCATCGTATCCGCCGCCCGCACCGCGGTCGGCAAGTTTGGCGGCTCGCTGGC
T3    rAspValValIleValSerAlaAlaArgThrAlaValGlyLysPheGlyGlySerLeuAl

2761 CAAGATCCCGGCACCGGAACTGGGTGCCGTGGTCATCAAGGCCGCGCTGGAGCGCGCCGG
T23   aLysIleProAlaProGluLeuGlyAlaValValIleLysAlaAlaLeuGluArgAlaGl

2821 CGTCAAGCCGGAGCAGGTGAGCGAAGTCATCATGGGCCAGGTGCTGACCGCCGGTTCGGG
T43   yValLysProGluGlnValSerGluValIleMetGlyGlnValLeuThrAlaGlySerGl

2881 CCAGAACCCCGCACGCCAGGCCGCGATCAAGGCCGGCCTCGGCGCGATGGTGCCGGCCAT
T63   yGlnAsnProAlaArgGlnAlaAlaIleLysAlaGlyLeuGlyAlaMetValProAlaMe

2941 GACCATCAACAAGGTGTGCGGCTCGGGCCTGAAGGCCGTGATGCTGGCCGCCAACGCGAT
T83   tThrIleAsnLysValCysGlySerGlyLeuLysAlaValMetLeuAlaAlaAsnAlaIl

3001 CATGGCGGGCGACGCCGAGATCGTGGTGGCCGGCGGCCAGGAAAACATGAGCGCCGCCCC
T103  eMetAlaGlyAspAlaGluIleValValAlaGlyGlyGlnGluAsnMetSerAlaAlaPr

3061 GCACGTGCTGCCGGGCTCGCGCGATGGTTTCCGCATGGGCGATGCCAAGCTGGTCGACAC
T123  oHisValLeuProGlySerArgAspGlyPheArgMetGlyAspAlaLysLeuValAspTh

3121 CATGATCGTCGACGGCCTGTGGGACGTGTACAACCAGTACCACATGGGCATCACCGCCGA
T143  rMetIleValAspGlyLeuTrpAspValTyrAsnGlnTyrHisMetGlyIleThrAlaGl

3181 GAACGTGGCCAAGGAATACGGCATCACACGCGAGGCGCAGGATGAGTTCGCCGTCGGCTC
T163  uAsnValAlaLysGluTyrGlyIleThrArgGluAlaGlnAspGluPheAlaValGlySe

3241 GCAGAACAAGGCCGAAGCCGCGCAGAAGGCCGGCAAGTTTGACGAAGAGATCGTCCCGGT
T183  rGlnAsnLysAlaGluAlaAlaGlnLysAlaGlyLysPheAspGluGluIleValProVa

3301 GCTGATCCCGCAGCGCAAGGGCGACCCGGTGGCCTTCAAGACCGACGAGTTCGTGCGCCA
T203  lLeuIleProGlnArgLysGlyAspProValAlaPheLysThrAspGluPheValArgGl

3361 GGGCGCCACGCTGGACAGCATGTCCGGCCTCAAGCCCGCCTTCGACAAGGCCGGCACGGT
T223  nGlyAlaThrLeuAspSerMetSerGlyLeuLysProAlaPheAspLysAlaGlyThrVa

3421 GACCGCGGCCAACGCCTCGGGCCTGAACGACGGCGCCGCCGCGGTGGTGGTGATGTCGGC
T243  lThrAlaAlaAsnAlaSerGlyLeuAsnAspGlyAlaAlaAlaValValValMetSerAl

3481 GGCCAAGGCCAAGGAACTGGGCCTGACCCCGCTGGCCACGATCAAGAGCTATGCCAACGC
T263  aAlaLysAlaLysGluLeuGlyLeuThrProLeuAlaThrIleLysSerTyrAlaAsnAl
```

```
3541 CGGTGTCGATCCCAAGGTGATGGGCATGGGCCCGGTGCCGGCCTCCAAGCGCGCCCTGTC
T283 aGlyValAspProLysValMetGlyMetGlyProValProAlaSerLysArgAlaLeuSe

3601 GCGCGCCGAGTGGACCCCGCAAGACCTGGACCTGATGGAGATCAACGAGGCCTTTGCCGC
T303 rArgAlaGluTrpThrProGlnAspLeuAspLeuMetGluIleAsnGluAlaPheAlaAl

3661 GCAGGCGCTGGCGGTGCACCAGCAGATGGGCTGGGACACCTCCAAGGTCAATGTGAACGG
T323 aGlnAlaLeuAlaValHisGlnGlnMetGlyTrpAspThrSerLysValAsnValAsnGl

3721 CGGCGCCATCGCCATCGGCCACCCGATCGGCGCGTCGGGCTGCCGTATCCTGGTGACGCT
T343 yGlyAlaIleAlaIleGlyHisProIleGlyAlaSerGlyCysArgIleLeuValThrLe

3781 GCTGCACGAGATGAAGCGCCGTGACGCGAAGAAGGGCCTGGCCTCGCTGTGCATCGGCGG
T363 uLeuHisGluMetLysArgArgAspAlaLysLysGlyLeuAlaSerLeuCysIleGlyGl

3841 CGGCATGGGCGTGGCGCTGGCAGTCGAGCGCAAATAAGGAAGGGGTTTTCCGGGGCCGCG
T383 yGlyMetGlyValAlaLeuAlaValGluArgLys*

3901 CGCGGTTGGCGCGGACCCGGCGACGATAACGAAGCCAATCAAGGAGTGGACATGACTCAG
R1                                                    MetThrGln

3961 CGCATTGCGTATGTGACCGGCGGCATGGGTGGTATCGGAACCGCCATTTGCCAGCGGCTG
R4   ArgIleAlaTyrValThrGlyGlyMetGlyGlyIleGlyThrAlaIleCysGlnArgLeu

4021 GCCAAGGATGGCTTTCGTGTGGTGGCCGGTTGCGGCCCCAACTCGCCGCGCCGCGAAAAG
R24  AlaLysAspGlyPheArgValValAlaGlyCysGlyProAsnSerProArgArgGluLys

4081 TGGCTGGAGCAGCAGAAGGCCCTGGGCTTCGATTTCATTGCCTCGGAAGGCAATGTGGCT
R44  TrpLeuGluGlnGlnLysAlaLeuGlyPheAspPheIleAlaSerGluGlyAsnValAla

4141 GACTGGGACTCGACCAAGACCGCATTCGACAAGGTCAAGTCCGAGGTCGGCGAGGTTGAT
R64  AspTrpAspSerThrLysThrAlaPheAspLysValLysSerGluValGlyGluValAsp

4201 GTGCTGATCAACAACGCCGGTATCACCCGCGACGTGGTGTTCCGCAAGATGACCCGCGCC
R84  ValLeuIleAsnAsnAlaGlyIleThrArgAspValValPheArgLysMetThrArgAla

4261 GACTGGGATGCGGTGATCGACACCAACCTGACCTCGCTGTTCAACGTCACCAAGCAGGTG
R104 AspTrpAspAlaValIleAspThrAsnLeuThrSerLeuPheAsnValThrLysGlnVal

4321 ATCGACGGCATGGCCGACCGTGGCTGGGGCCGCATCGTCAACATCTCGTCGGTGAACGGG
R124 IleAspGlyMetAlaAspArgGlyTrpGlyArgIleValAsnIleSerSerValAsnGly
```

```
4381 CAGAAGGGCCAGTTCGGCCAGACCAACTACTCCACCGCCAAGGCCGGCCTGCATGGCTTC
R144 GlnLysGlyGlnPheGlyGlnThrAsnTyrSerThrAlaLysAlaGlyLeuHisGlyPhe

4441 ACCATGGCACTGGCGCAGGAAGTGGCGACCAAGGGCGTGACCGTCAACACGGTCTCTCCG
R164 ThrMetAlaLeuAlaGlnGluValAlaThrLysGlyValThrValAsnThrValSerPro

4501 GGCTATATCGCCACCGACATGGTCAAGGCGATCCGCCAGGACGTGCTCGACAAGATCGTC
R184 GlyTyrIleAlaThrAspMetValLysAlaIleArgGlnAspValLeuAspLysIleVal

4561 GCGACGATCCCGGTCAAGCGCCTGGGCCTGCCGGAAGAGATCGCCTCGATCTGCGCCTGG
R204 AlaThrIleProValLysArgLeuGlyLeuProGluGluIleAlaSerIleCysAlaTrp

4621 TTGTCGTCGGAGGAGTCCGGTTTCTCGACCGGCGCCGACTTCTCGCTCAACGGCGGCCTG
R224 LeuSerSerGluGluSerGlyPheSerThrGlyAlaAspPheSerLeuAsnGlyGlyLeu

4681 CATATGGGCTGACCTGCCGGCCTGGTTCAACCAGTCGGCAGCCGGCGCTGGCGCCCGCGT
R244 HisMetGly*

4741 ATTGCGGTGCAGCCAGCGCGGCGCACAAGGCGGCGGGCGTTTCGTTTCGCCGCCCGTTTC

4801 GCGGCAAGGCCCGCGAATCGTTTCTGCCCGCGCGGCNTTCCTCGCTTTTTGCGCCAATTC

4861 ACCGGGTTTTCCTTTAAGCCCCGTCGCTTTTCTTAGTGCCTTGTTGGGCATAGAATCAGG

4921 GCAGCGGCGCAGCCAGCACCATGTTCGTGCAGCGCGGCCCTCGCGGGGGCGAGGCTGCAG
```

CLAIMS:

1. A transgenic plant material containing foreign DNA leading to the production of a polyhydroxyalkanoate, preferably wherein the polyhydroxyalkanoate is polyhydroxybutyrate.

2. The plant material of Claim 2 wherein coding sequence of the DNA and RNA for the production of the enzymes leading to polyhydroxybutyrate synthesis are as shown in SEQ ID NO: 1.

3. A transgenic plant material containing foreign DNA encoding a peptide which exhibits 3-ketothiolase activity, preferably wherein the DNA is an open reading frame between 2696 and 3877 of SEQ ID NO: 1.

4. A transgenic plant material containing foreign DNA encoding acetoacetyl-CoA reductase activity, preferably wherein the DNA is an open reading frame between 3952 and 4692 of SEQ ID NO: 1.

5. A transgenic plant material containing foreign DNA encoding a polypeptide which exhibits PHA synthase activity.

6. A transgenic plant material containing foreign DNA encoding one or more enzymes leading to the synthesis of polyhydroxyalkanoate from hydroxyalkyl-CoA, preferably wherein the DNA is an open reading frame between 842 and 2611 of SEQ ID NO: 1.

7. A transgenic plant material containing foreign DNA encoding one or more enzymes which catalyze synthesis of hydroxyalkyl-CoA.

8. A transgenic plant material containing foreign DNA encoding one or more enzymes leading to production of acetoacetyl-CoA from products encoded by the foreign DNA.

9. The plant material of Claim 1, 2, 3, 4, 5, 6, 7 or 8 as a seed or propagule of the seed.

10. A method for introducing foreign DNA encoding polypeptides leading to the synthesis of a polyhydroxyalkanoate into a plant which comprises mating by sexual fertilization two plants which do not produce polyhydroxyalkanoate, each containing foreign DNA encoding one or more different enzymes in a pathway leading to polymerization of hydroxyalkyl-CoA by polyhydroxyalkanoate synthase to produce the plant encoding the polyhydroxyalkanoate, preferably wherein the polyhydroxyalkanoate is polyhydroxybutyrate.

11. The method of Claim 10 wherein the polyhydroxyalkanoate is in granules in cells of the plant.

12. A gene segment as contained in a seed deposited as ATCC 75042 containing DNA encoding the 3-ketothiolase gene.

13. A plant containing the gene segment of Claim 12, preferably wherein the plant is Arabidopsis thaliana.

14. A gene segment as contained in a seed deposited as ATCC 75044 containing DNA encoding the acetoacetyl-CoA reductase gene.

15. A plant containing the gene segment of Claim 14, preferably wherein the plant is Arabidopsis thaliana.

16. A gene segment as contained in a seed deposited as ATCC 75043 containing DNA encoding the PHB synthase gene.

17. A plant containing the gene segment of Claim 16, preferably wherein the plant is Arabidopsis thaliana.

18. A transgenic plant material containing foreign DNA encoding one or more enzymes leading to production of 3-hydroxybutyrl-CoA from products encoded by the foreign DNA.

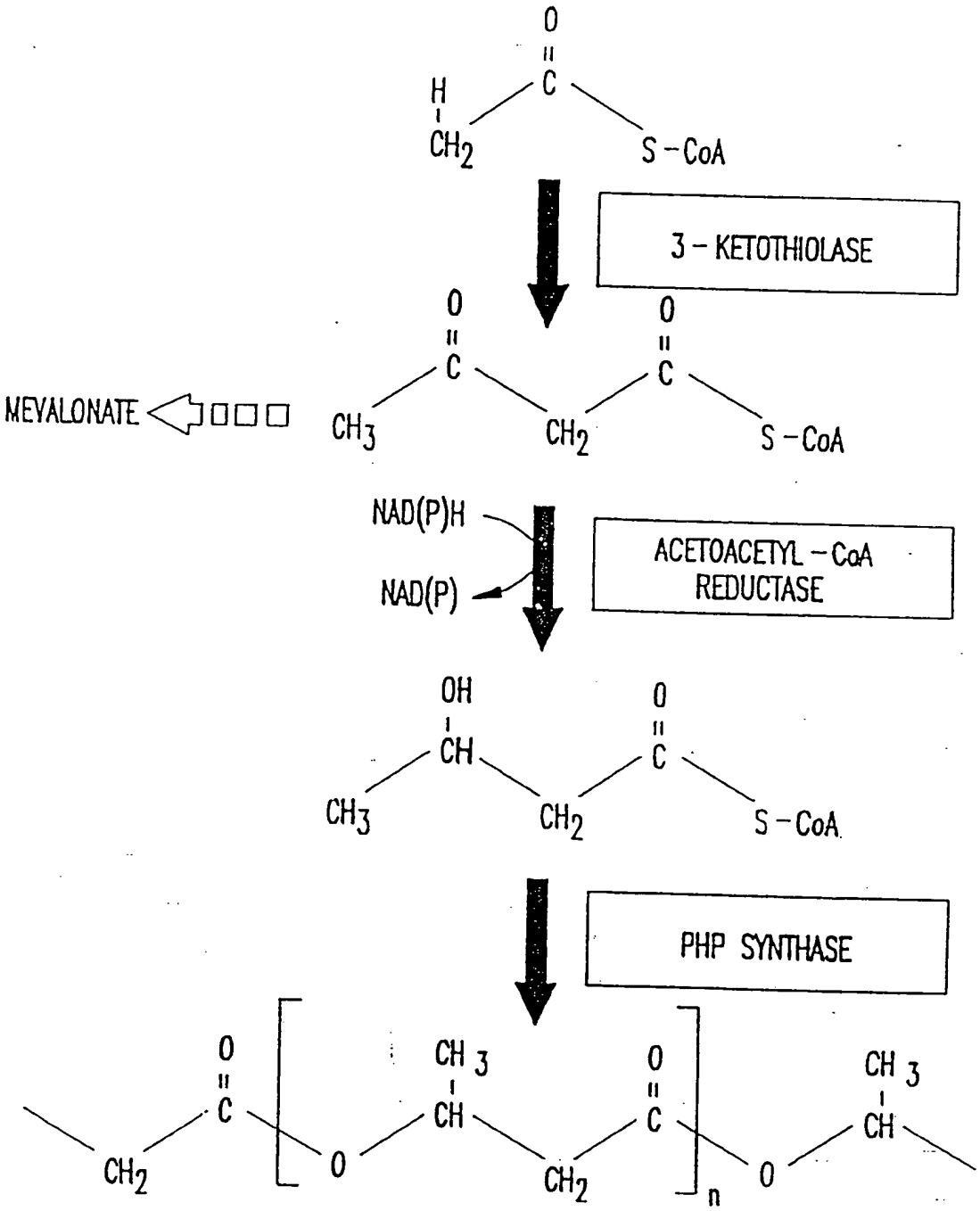

3 - KETOTHIOLASE
MEVALONATE
NAD(P)H
NAD(P)
ACETOACETYL - CoA REDUCTASE
PHP SYNTHASE

FIG. 1

```
1    CCCGGGCAAGTACCTTGCCGACATCTATGCGCTGGCGCGCACGCGCCTGGCGCGCGCCGG
61   CTGTACCGAGGTCTACGGCGGCGACGCCTGCACCGTGGCCGACGCCGGTCGCTTCTACTC
121  CTATCGGCGCGATGGCGTGACCGGCCGCATGGCCAGCCTGGTCTGGCTGGCGGACTGAGC
                                    DdeI
181  CCGCCGCTGCCTCACTCGTCCTTGCCCCTGGCCGCCTGCGCGCGCTCGGCTTCAGCCTTG
241  CGTCGGCGGCGGCCGGGCGTGCCCATGATGTAGAGCACCACGCCACCGGCGCCATGCCAT
301  ACATCAGGAAGGTGGCAACGCCTGCCACCACGTTGTGCTCGGTGATCGCCATCATCAGCG
361  CCACGTAGAGCCAGCCAATGGCCACGATGTACATCAAAAATTCATCCTTCTCGCCTATGC
421  TCTGGGGCCTCGGCAGATGCGAGCGCTGCATACCGTCCGGTAGGTCGGGAAGCGTGCAGT
481  GCCGAGGCGGATTCCCGCATTGACAGCGCGTGCGTTGCAAGGCAACAATGGACTCAAATG
541  TCTCGGAATCGCTGACGATTCCCAGGTTTCTCCGGCAAGCATAGCGCATGGCGTCTCCAT
601  GCGAGAATGTCGCGCTTGCCGGATAAAAGGGGAGCCGCTATCGGAATGGACGCAAGCCAC
661  GGCCGCAGCAGGTGCGGTCGAGGGCTTCCAGCCAGTTCCAGGGCAGATGTGCCGGCAGAC
721  CCTCCCGCTTTGGGGGAGGCGCAAGCCGGGTCCATTCGGATAGCATCTCCCCATGCAAAG
```

FIG. 2A

```
                                                BstBI
781   TGCCGGCCAGGGCAATGCCCGGAGCCGGTTCGAATAGTGACGGCAGAGAGACAATCAAAT

841   CATGGCGACCGGCAAAGGCGCGGCAGCTTCCACGCAGGAAGGCAAGTCCCAACCATTCAA
S1    M  A  T  G  K  G  A  A  A  S  T  Q  E  G  K  S  Q  P  F  K

901   GGTCACGCCGGGGCCATTCGATCCAGCCACATGGCTGGAATGGTCCCGCCAGTGGCAGGG
S21    V  T  P  G  P  F  D  P  A  T  W  L  E  W  S  R  Q  W  Q  G

961   CACTGAAGGCAACGGCCACGCGGCCGCGTCCGGCATTCCGGGCCTGGATGCGCTGGCAGG
S41    T  E  G  N  G  H  A  A  A  S  G  I  P  G  L  D  A  L  A  G

                                                                DdeI
1021  CGTCAAGATCGCGCCGGCGCAGCTGGGTGATATCCAGCAGCGCTACATGAAGGACTTCTC
S61    V  K  I  A  P  A  Q  L  G  D  I  Q  Q  R  Y  M  K  D  F  S

1081  AGCGCTGTGGCAGGCCATGGCCGAGGGCAAGGCCGAGGCCACCGGTCCGCTGCACGACCG
S81    A  L  W  Q  A  M  A  E  G  K  A  E  A  T  G  P  L  H  D  R

1141  GCGCTTCGCCGGCGACGCATGGCGCACCAACCTCCCATATCGCTTCGCTGCCGCGTTCTA
S101   R  F  A  G  D  A  W  R  T  N  L  P  Y  R  F  A  A  A  F  Y

1201  CCTGCTCAATGCGCGCGCCTTGACCGAGCTGGCCGATGCCGTCGAGGCCGATGCCAAGAC
S121   L  L  N  A  R  A  L  T  E  L  A  D  A  V  E  A  D  A  K  T
```

FIG. 2B

```
1261 CCGCCAGCGCATCCGCTTCGCGATCTCGCAATGGGTCGATGCGATGTCGCCCGCCAACTT
S141  R  Q  R  I  R  F  A  I  S  Q  W  V  D  A  M  S  P  A  N  F

1321 CCTTGCCACCAATCCCGAGGCGCAGCGCCTGCTGATCGAGTCGGGCGGCGAATCGCTGCG
S161  L  A  T  N  P  E  A  Q  R  L  L  I  E  S  G  G  E  S  L  R

1381 TGCCGGCGTGCGCAACATGATGGAAGACCTGACACGCGGCAAGATCTCGCAGACCGACGA
S181  A  G  V  R  N  M  M  E  D  L  T  R  G  K  I  S  Q  T  D  E

1441 GAGCGCGTTTGAGGTCGGCCGCAATGTCGCGGTGACCGAAGGCGCCGTGGTCTTCGAGAA
S201  S  A  F  E  V  G  R  N  V  A  V  T  E  G  A  V  V  F  E  N

1501 CGAGTACTTCCAGCTGTTGCAGTACAAGCCGCTGACCGACAAGGTGCACGCGCGCCCGCT
S221  E  Y  F  Q  L  L  Q  Y  K  P  L  T  D  K  V  H  A  R  P  L

                                          PstI
1561 GCTGATGGTGCCGCCGTGCATCAACAAGTACTACATCCTGGACCTGCAGCCGGAGAGCTC
S241  L  M  V  P  P  C  I  N  K  Y  Y  I  L  D  L  Q  P  E  S  S

1621 GCTGGTGCGCCATGTGGTGGAGCAGGGACATACGGTGTTTCTGGTGTCGTGGCGCAATCC
S261  L  V  R  H  V  V  E  Q  G  H  T  V  F  L  V  S  W  R  N  P

1681 GGACGCCAGCATGGCCGGCAGCACCTGGGACGACTACATCGAGCACGCGGCCATCCGCGC
S281  D  A  S  M  A  G  S  T  W  D  D  Y  I  E  H  A  A  I  R  A
```

FIG. 2C

```
1741 CATCGAAGTCGCGCGCGACATCAGCGGCCAGGACAAGATCAACGTGCTCGGCTTCTGCGT
S301  I  E  V  A  R  D  I  S  G  Q  D  K  I  N  V  L  G  F  C  V

1801 GGGCGGCACCATTGTCTCGACCGCGCTGGCGGTGCTGGCCGCGCGCGCCGAGCACCCGGC
S321  G  G  T  I  V  S  T  A  L  A  V  L  A  A  R  G  E  H  P  A

1861 CGCCAGCGTCACGCTGCTGACCACGCTGCTGGACTTTGCCGACACGGGCATCCTCGACGT
S341  A  S  V  T  L  L  T  T  L  L  D  F  A  D  T  G  I  L  D  V

1921 CTTTGTCGACGAGGGCCATGTGCAGTTGCGCGAGGCCACGCTGGGCGGCGGCGCCGGCGC
S361  F  V  D  E  G  H  V  Q  L  R  E  A  T  L  G  G  G  A  G  A

1981 GCCGTGCGCGCTGCTGCGCGGCCTTGAGCTGGCCAATACCTTCTCGTTCTTGCGCCCGAA
S381  P  C  A  L  L  R  G  L  E  L  A  N  T  F  S  F  L  R  P  N

2041 CGACCTGGTGTGGAACTACGTGGTCGACAACTACCTGAAGGGCAACACGCCGGTGCCGTT
S401  D  L  V  W  N  Y  V  V  D  N  Y  L  K  G  N  T  P  V  P  F

2101 CGACCTGCTGTTCTGGAACGGCGACGCCACCAACCTGCCGGGGCCGTGGTACTGCTGGTA
S421  D  L  L  F  W  N  G  D  A  T  N  L  P  G  P  W  Y  C  W  Y

                    PstI
2161 CCTGCGCCACACCTACCTGCAGAACGAGCTCAAGGTACCGGGCAAGCTGACCGTGTGCGG
S441  L  R  H  T  Y  L  Q  N  E  L  K  V  P  G  K  L  T  V  C  G
```

FIG. 2D

```
2221 CGTGCCGGTGGACCTGGCCAGCATCGACGTGCCGACCTATATCTACGGCTCGCGCGAAGA
S461  V  P  V  D  L  A  S  I  D  V  P  T  Y  I  Y  G  S  R  E  D

2281 CCATATCGTGCCGTGGACCGCGGCCTATGCCTCGACCGCGCTGCTGGCGAACAAGCTGCG
S481  H  I  V  P  W  T  A  A  Y  A  S  T  A  L  L  A  N  K  L  R

2341 CTTCGTGCTGGGTGCGTCGGGCCATATCGCCGGTGTGATCAACCCGCCGGCCAAGAACAA
S501  F  V  L  G  A  S  G  H  I  A  G  V  I  N  P  P  A  K  N  K

2401 GCGCAGCCACTGGACTAACGATGCGCTGCCGGAGTCGCCGCAGCAATGGCTGGCCGGCGC
S521  R  S  H  W  T  N  D  A  L  P  E  S  P  Q  Q  W  L  A  G  A

2461 CATCGAGCATCACGGCAGCTGGTGGCCGGACTGGACCGCATGGCTGGCCGGGCAGGCCGG
S541  I  E  H  H  G  S  W  W  P  D  W  T  A  W  L  A  G  Q  A  G

2521 CGCGAAACGCGCCGCGCCCGCCAACTATGGCAATGCGCGCTATCGCGCAATCGAACCCGC
S561  A  K  R  A  A  P  A  N  Y  G  N  A  R  Y  R  A  I  E  P  A

2581 GCCTGGGCGATACGTCAAAGCCAAGGCATGACGCTTGCATGAGTGCCGGCGTGCGTCATG
S581  P  G  R  Y  V  K  A  K  A  *

                    PstI
2641 CACGGCGCCGGCAGGCCTGCAGGTTCCCTCCCGTTTCCATTGAAAGGACTACACAATGAC
T1                                                          M  T
```

FIG. 2E

```
          Tth1111
2701 TGACGTTGTCATCGTATCCGCCGCCCGCACCGCGGTCGGCAAGTTTGGCGGCTCGCTGGC
T3     D  V  V  I  V  S  A  A  R  T  A  V  G  K  F  G  G  S  L  A

2761 CAAGATCCCGGCACCGGAACTGGGTGCCGTGGTCATCAAGGCCGCGCTGGAGCGCGCCGG
T23    K  I  P  A  P  E  L  G  A  V  V  I  K  A  A  L  E  R  A  G

2821 CGTCAAGCCGGAGCAGGTGAGCGAAGTCATCATGGGCCAGGTGCTGACCGCCGGTTCGGG
T43    V  K  P  E  Q  V  S  E  V  I  M  G  Q  V  L  T  A  G  S  G

2881 CCAGAACCCCCGCACGCCAGGCCGCGATCAAGGCCGGCCTCGGCGCGATGGTGCCGGCCAT
T63    Q  N  P  A  R  Q  A  A  I  K  A  G  L  G  A  M  V  P  A  M

2941 GACCATCAACAAGGTGTGCGGCTCGGGCCTGAAGGCCGTGATGCTGGCCGCCAACGCGAT
T83    T  I  N  K  V  C  G  S  G  L  K  A  V  M  L  A  A  N  A  I

3001 CATGGCGGGCGACGCCGAGATCGTGGTGGCCGGCGGCCAGGAAAACATGAGCGCCGCCCC
T103   M  A  G  D  A  E  I  V  V  A  G  G  Q  E  N  M  S  A  A  P

3061 GCACGTGCTGCCGGGCTCGCGCGATGGTTTCCGCATGGGCGATGCCAAGCTGGTCGACAC
T123   H  V  L  P  G  S  R  D  G  F  R  M  G  D  A  K  L  V  D  T

3121 CATGATCGTCGACGGCCTGTGGGACGTGTACAACCAGTACCACATGGGCATCACCGCCGA
T143   M  I  V  D  G  L  W  D  V  Y  N  Q  Y  H  M  G  I  T  A  E
```

FIG. 2F

```
3181 GAACGTGGCCAAGGAATACGGCATCACACGCGAGGCGCAGGATGAGTTCGCCGTCGGCTC
T163   N   V   A   K   E   Y   G   I   T   R   E   A   Q   D   E   F   A   V   G   S

3241 GCAGAACAAGGCCGAAGCCGCGCAGAAGGCCGGCAAGTTTGACGAAGAGATCGTCCCGGT
T183   Q   N   K   A   E   A   A   Q   K   A   G   K   F   D   E   E   I   V   P   V

3301 GCTGATCCCGCAGCGCAAGGGCGACCCGGTGGCCTTCAAGACCGACGAGTTCGTGCGCCA
T203   L   I   P   Q   R   K   G   D   P   V   A   F   K   T   D   E   F   V   R   Q

3361 GGGCGCCACGCTGGACAGCATGTCCGGCCTCAAGCCCGCCTTCGACAAGGCCGGCACGGT
T223   G   A   T   L   D   S   M   S   G   L   K   P   A   F   D   K   A   G   T   V

3421 GACCGCGGCCAACGCCTCGGGCCTGAACGACGGCGCCGCCGCGGTGGTGGTGATGTCGGC
T243   T   A   A   N   A   S   G   L   N   D   G   A   A   A   V   V   V   M   S   A

3481 GGCCAAGGCCAAGGAACTGGGCCTGACCCCGCTGGCCACGATCAAGAGCTATGCCAACGC
T263   A   K   A   K   E   L   G   L   T   P   L   A   T   I   K   S   Y   A   N   A

3541 CGGTGTCGATCCCAAGGTGATGGGCATGGGCCCGGTGCCGGCCTCCAAGCGCGCCCTGTC
T283   G   V   D   P   K   V   M   G   M   G   P   V   P   A   S   K   R   A   L   S

3601 GCGCGCCGAGTGGACCCCGCAAGACCTGGACCTGATGGAGATCAACGAGGCCTTTGCCGC
T303   R   A   E   W   T   P   Q   D   L   D   L   M   E   I   N   E   A   F   A   A
```

FIG. 2G

```
3661 GCAGGCGCTGGCGGTGCACCAGCAGATGGGCTGGGACACCTCCAAGGTCAATGTGAACGG
T323   Q   A   L   A   V   H   Q   Q   M   G   W   D   T   S   K   V   N   V   N   G

3721 CGGCGCCATCGCCATCGGCCACCCGATCGGCGCGTCGGGCTGCCGTATCCTGGTGACGCT
T343   G   A   I   A   I   G   H   P   I   G   A   S   G   C   R   I   L   V   T   L

3781 GCTGCACGAGATGAAGCGCCGTGACGCGAAGAAGGGCCTGGCCTCGCTGTGCATCGGCGG
T363   L   H   E   M   K   R   R   D   A   K   K   G   L   A   S   L   C   I   G   G

3841 CGGCATGGGCGTGGCGCTGGCAGTCGAGCGCAAATAAGGAAGGGGTTTTCCGGGGCCGCG
T383   G   M   G   V   A   L   A   V   E   R   K   *

                                                                    DdeI
3901 CGCGGTTGGCGCGGACCCGGCGACGATAACGAAGCCAATCAAGGAGTGGACATGACTCAG
R1                                                          M   T   Q

3961 CGCATTGCGTATGTGACCGGCGGCATGGGTGGTATCGGAACCGCCATTTGCCAGCGGCTG
R4     R   I   A   Y   V   T   G   G   M   G   G   I   G   T   A   I   C   Q   R   L

4021 GCCAAGGATGGCTTTCGTGTGGTGGCCGGTTGCGGCCCCAACTCGCCGCGCCGCGAAAAG
R24    A   K   D   G   F   R   V   V   A   G   C   G   P   N   S   P   R   R   E   K

4081 TGGCTGGAGCAGCAGAAGGCCCTGGGCTTCGATTTCATTGCCTCGGAAGGCAATGTGGCT
R44    W   L   E   Q   Q   K   A   L   G   F   D   F   I   A   S   E   G   N   V   A
```

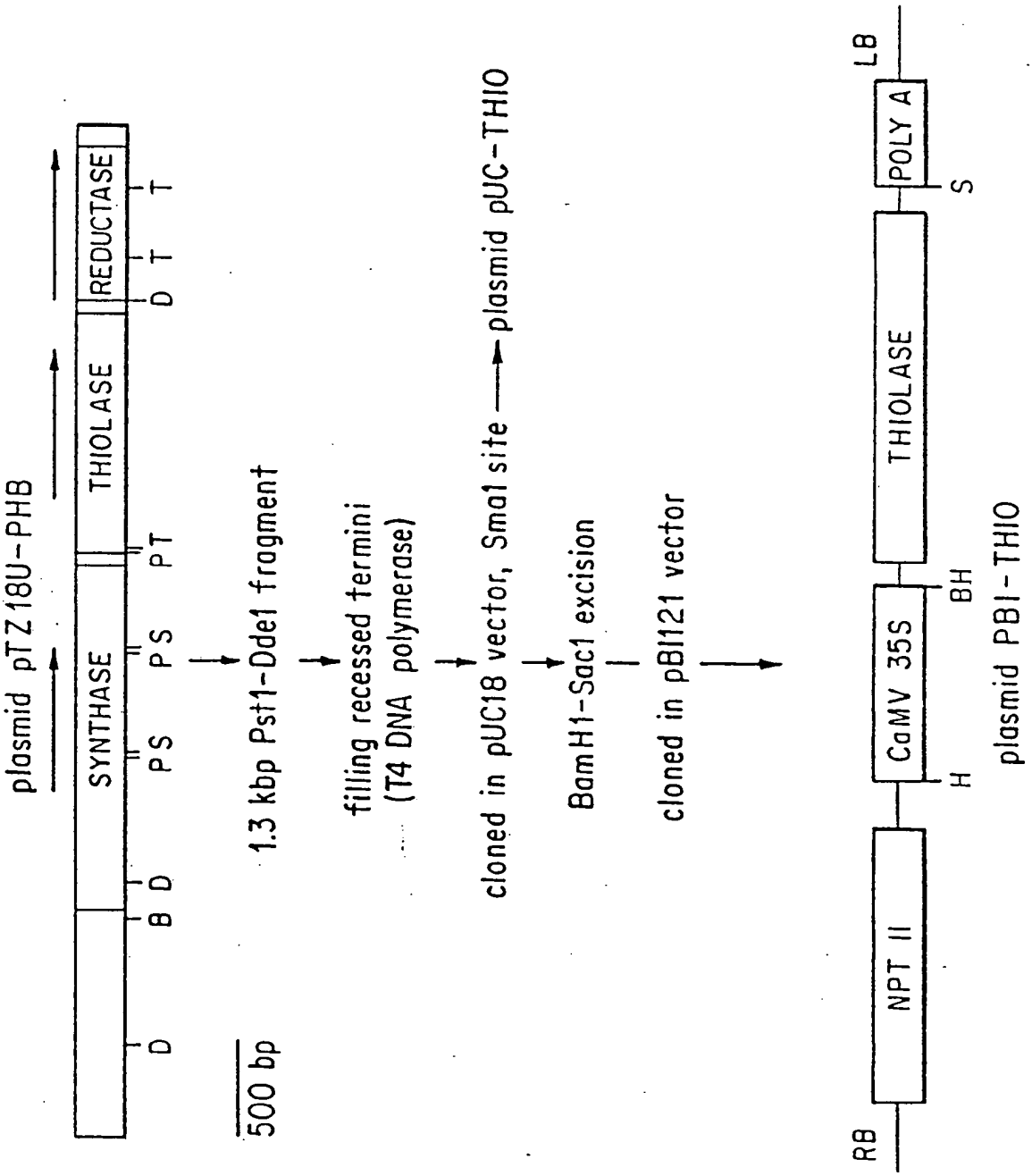
FIG. 2H

FIG. 2I

```
4621 TTGTCGTCGGAGGAGTCCGGTTCTCGACCGGCGCCGACTTCTCGCTCAACGGCGGCCTG
R224 L  S  S  E  E  S  G  F  S  T  G  A  D  F  S  L  N  G  G  L

4681 CATATGGGCTGACCTGCCGGCCTGGTTCAACCAGTCGGCAGCCGGCTGGCGCCCGCGT
R244 H  M  G  *

4741 ATTGCGGTGCAGCCAGCGCGGCGCACAAGGCGGGCGTTTCGTTTCGCCGCCCGTTTC

4801 GCGGCAAGGCCCGCGAATCGTTTCTGCCCGCGCGGCNTTCCTCGCTTTTTGCGCCAATTC
                    DdeI
4861 ACCGGGTTTTTCCTTTAAGCCCCGTCGCTTTTTCTTAGTGCCTTGTTGGGCATAGAATCAGG
                                                    PstI
4921 GCAGCGGCGCAGCCAGGCACCATGTTCGTGCAGCGCGGCCCTCGGGGGCGAGGCTGCAG
```

FIG. 2J

FIG. 3


plasmid pTZ18U-PHB
SYNTHASE
THIOLASE
REDUCTASE
D
B D
P S
P S
P T
D T T
500 bp
1.3 kbp Pst1-Dde1 fragment
filling recessed termini
(T4 DNA polymerase)
cloned in pUC18 vector, Sma1 site
plasmid pUC-THIO
BamH1-Sac1 excision
cloned in pBI121 vector
RB
NPT II
CaMV 35S
THIOLASE
POLY A
LB
H
BH
S
plasmid PBI-THIO

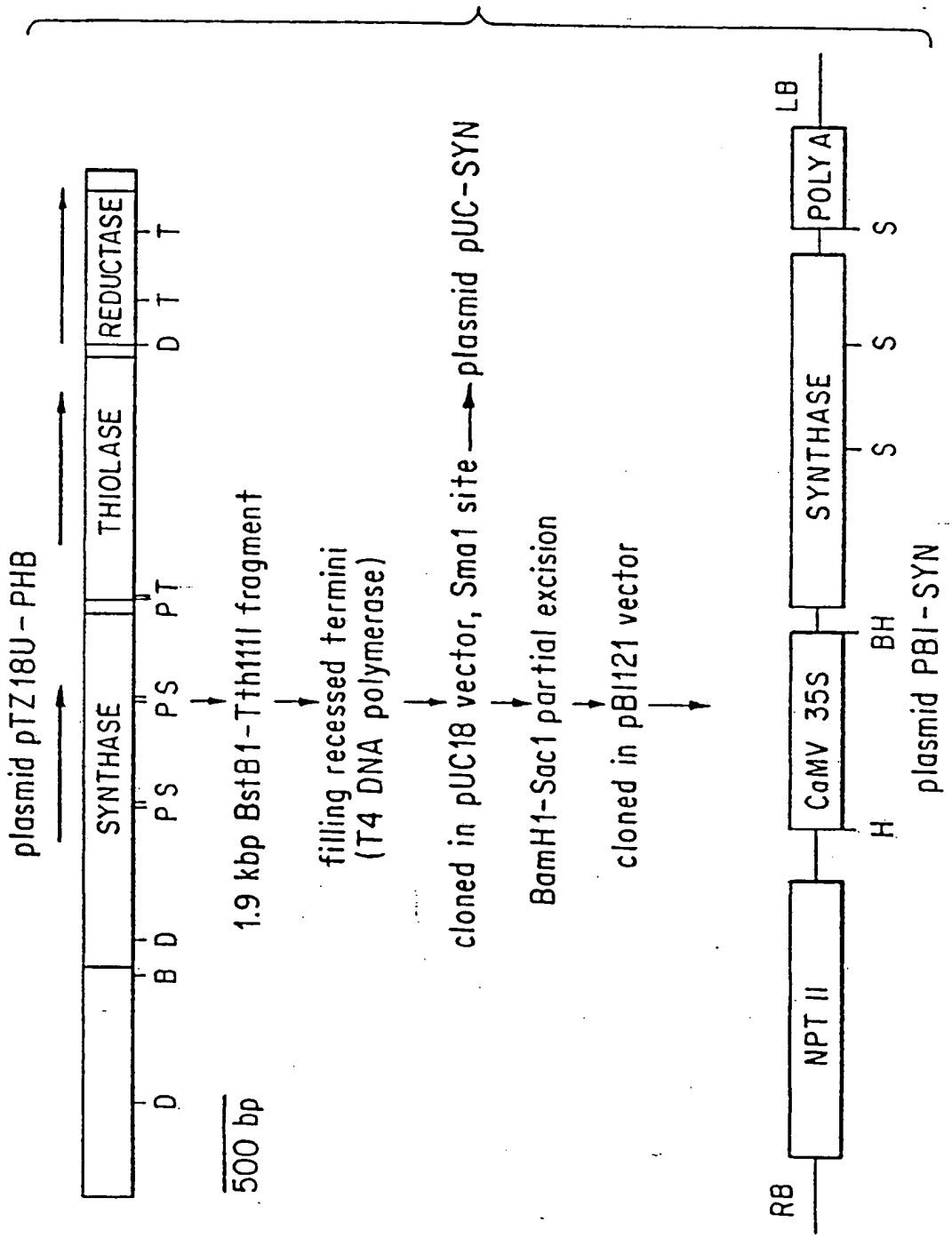

plasmid pTZ18U-PHB
SYNTHASE
THIOLASE
REDUCTASE
D B D P S P S P T D T T
500 bp
1.9 kbp BstB1-Tth111I fragment
filling recessed termini
(T4 DNA polymerase)
cloned in pUC18 vector, Sma1 site
plasmid pUC-SYN
BamH1-Sac1 partial excision
cloned in pBI121 vector
LB
POLY A
S S
SYNTHASE
S S
BH
CaMV 35S
H
NPT II
RB
plasmid PBI-SYN

FIG. 4

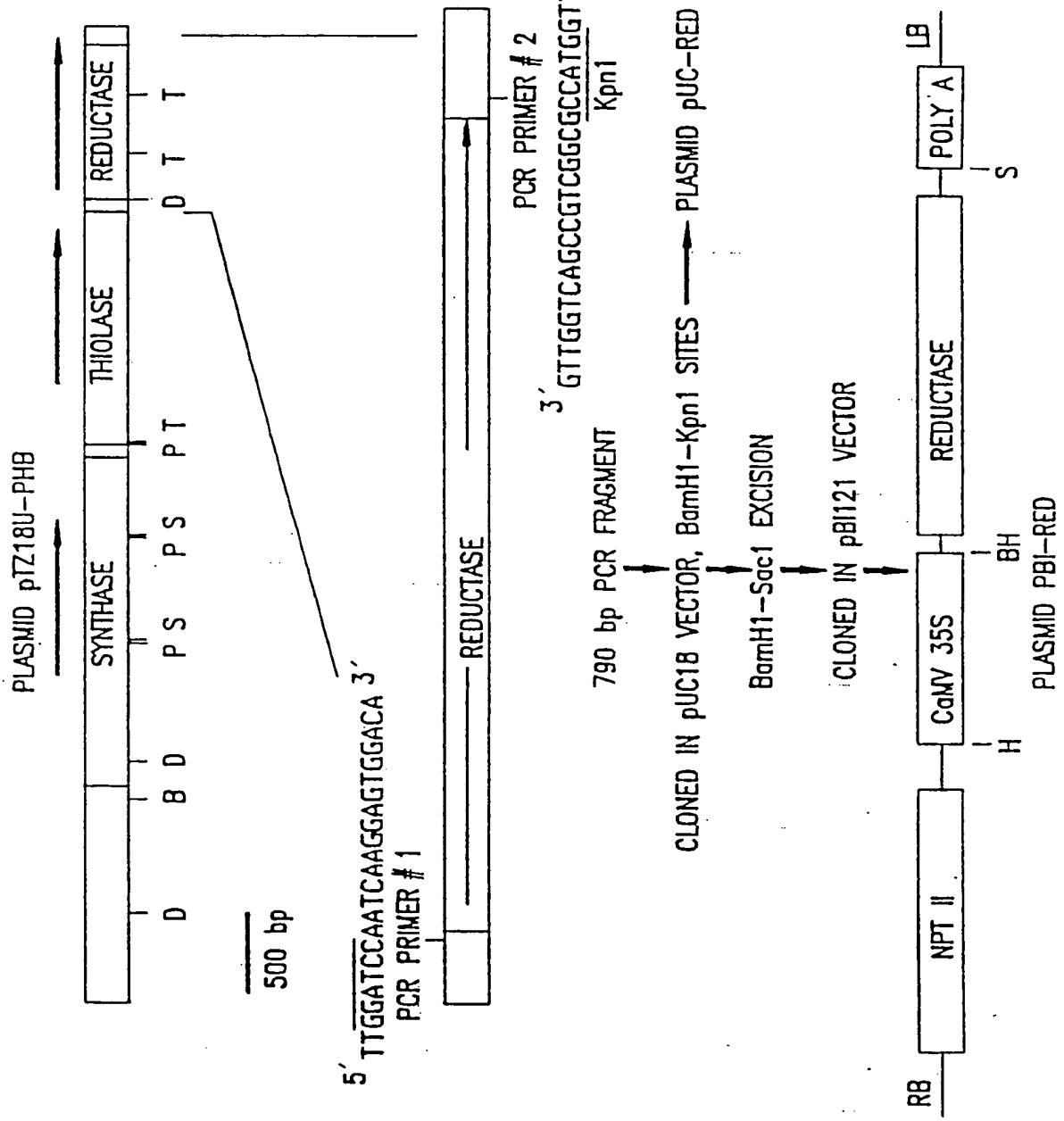

PLASMID pTZ18U-PHB
SYNTHASE
THIOLASE
REDUCTASE
D B D P S P S P T D T T
500 bp
5' TTGGATCCAATCAAGGAGTGGACA 3'
PCR PRIMER # 1
REDUCTASE
3' GTTGGTCAGCCGTCGGCGCCATGGTT 5'
PCR PRIMER # 2
Kpn1
790 bp PCR FRAGMENT
CLONED IN pUC18 VECTOR, BamH1-Kpn1 SITES
PLASMID pUC-RED
BamH1-Sac1 EXCISION
CLONED IN pBI121 VECTOR
RB
NPT II
CaMV 35S
H
BH
REDUCTASE
POLY A
S
LB
PLASMID PBI-RED

FIG.5

FIG.6A

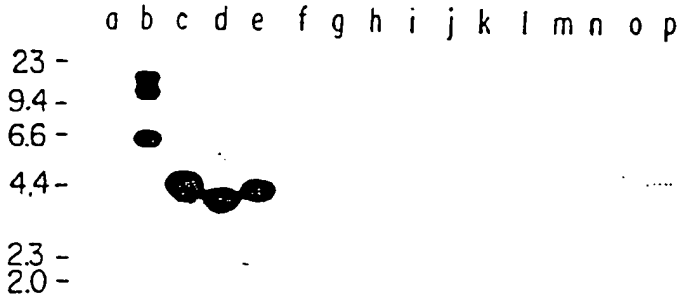


FIG.6B

FIG.6C

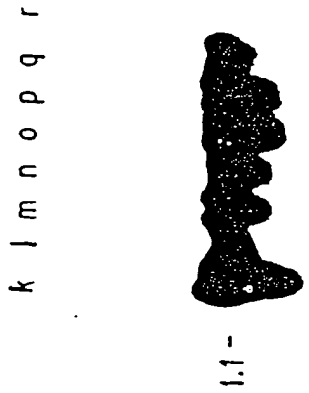


FIG. 7C

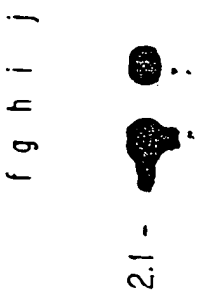


FIG. 7B

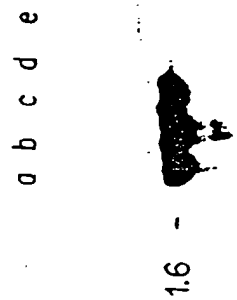


FIG. 7A

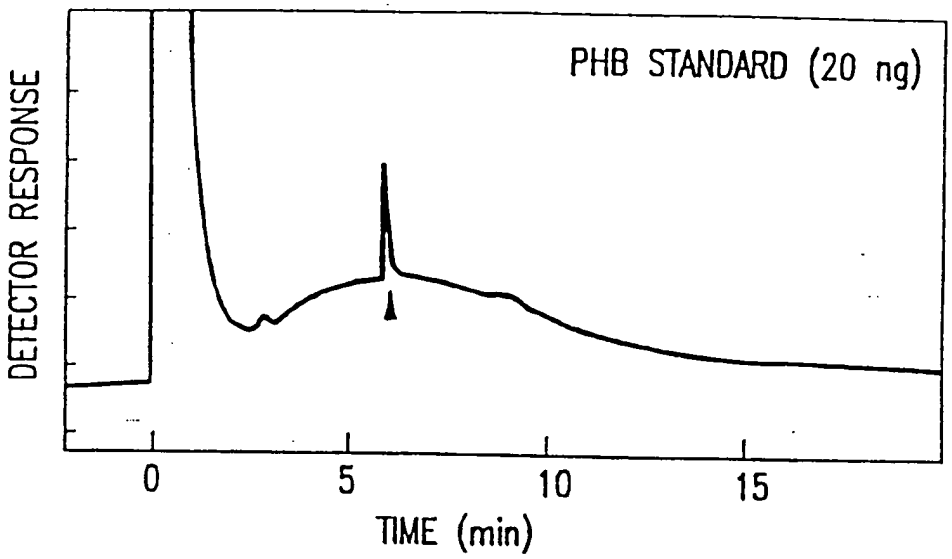


FIG.8A

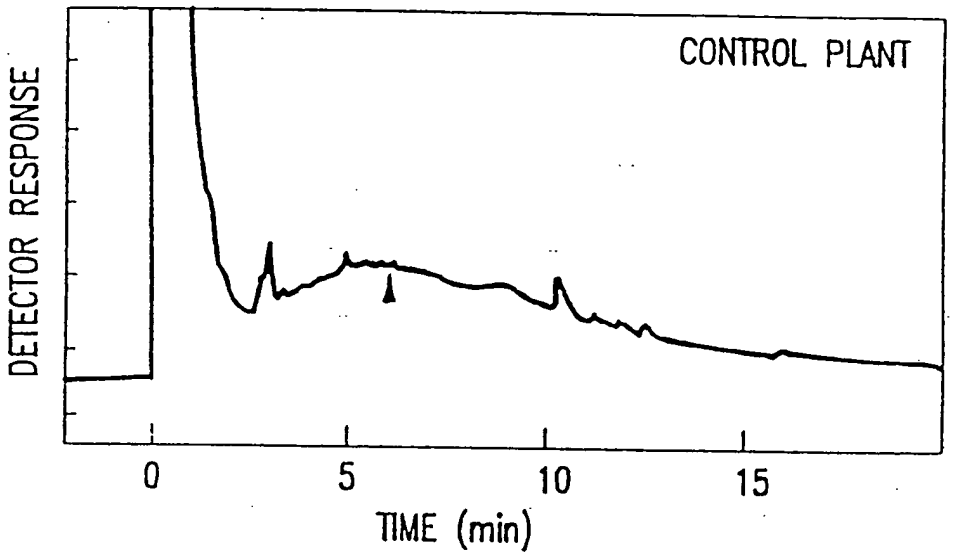


FIG.8B

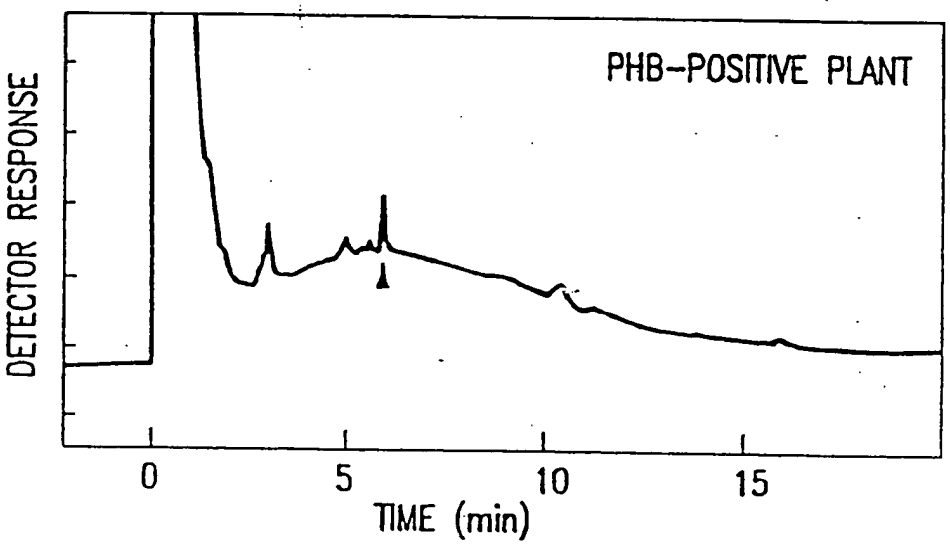


FIG.8C

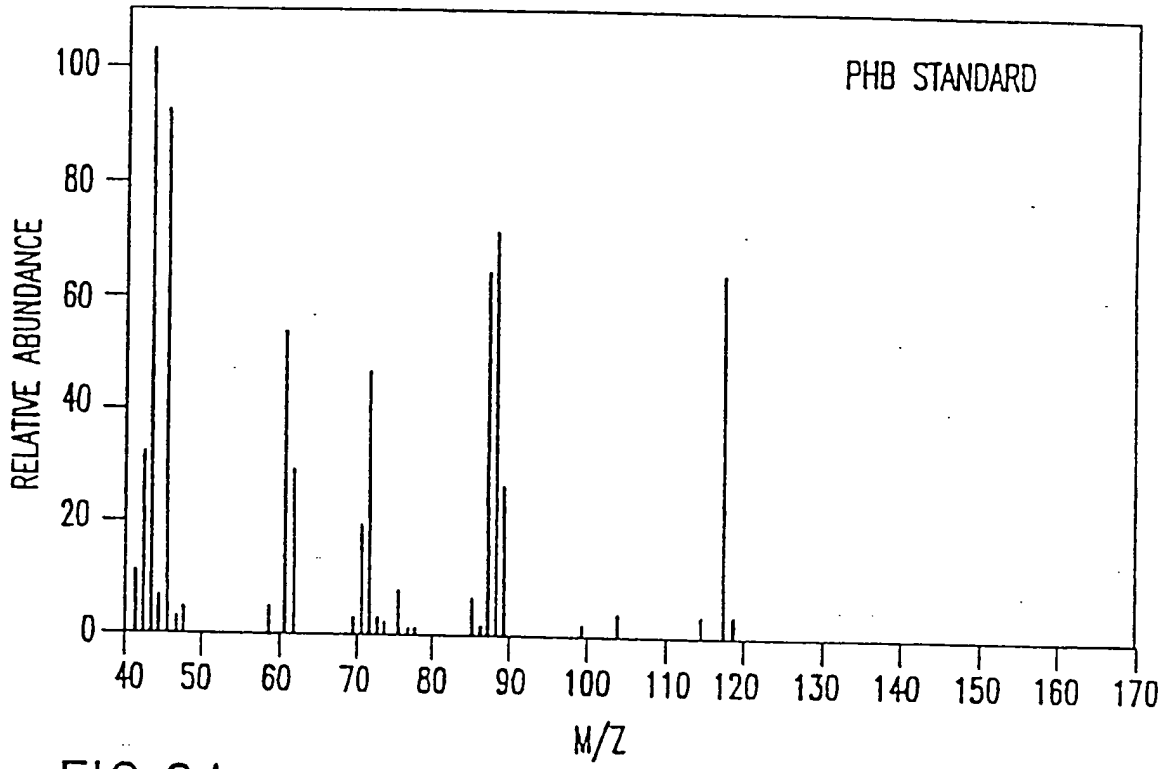

PHB STANDARD
RELATIVE ABUNDANCE
100
80
60
40
20
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170
M/Z

FIG.9A

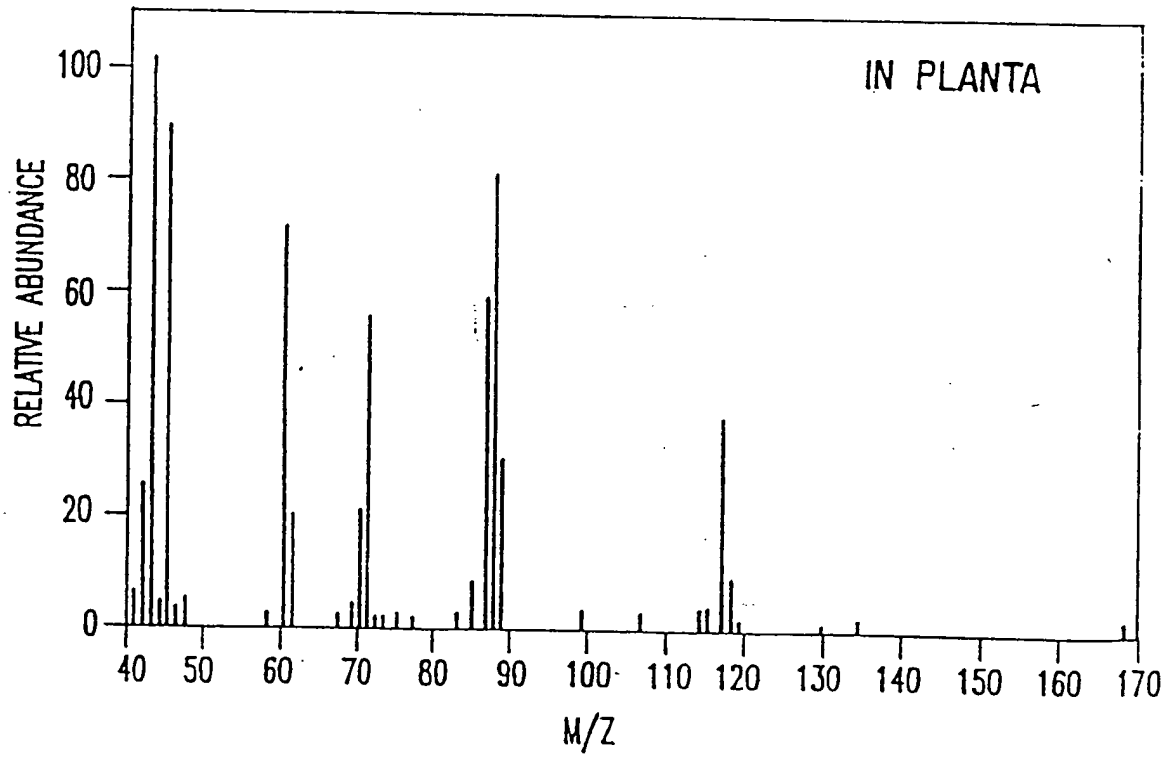

IN PLANTA
RELATIVE ABUNDANCE
100
80
60
40
20
0
40 50 60 70 80 90 100 110 120 130 140 150 160 170
M/Z

FIG.9B

FIG. 10A

FIG. 10B

FIG.10C

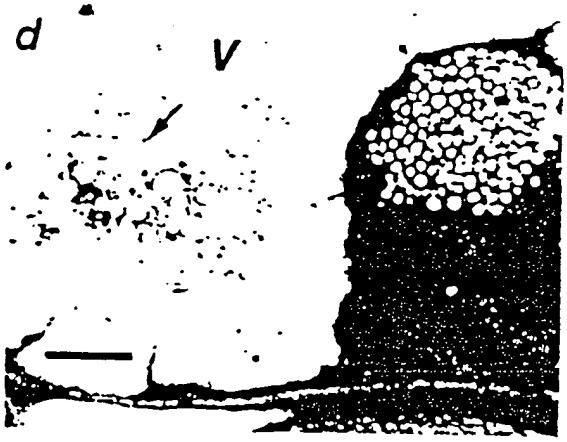


FIG.10D

FIG. 10E

FIG. 10F

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00; A01H 1 00
US CL :800/205, 250, 255; 435/172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205, 250, 255; 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PTOS APS, STN/BIOSIS,
search terms: polyhydroxybutyrate, polyhydroxyalkanoate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 245, issued 15 September 1989, Robert Pool, "In Search of the Plastic Potato", pages 1187-1189, see entire article.	1-18
Y	The Journal of Biological Chemistry, Volume 264, No. 26, issued 15 September 1989, O. P. Peoples et al., "Poly-beta-hydroxybutyrate Synthesis in Alcaligenes eutrophus H16", pages 15293-15297, especially page 15294.	1-4, 7-15, 18
Y	The Journal of Biological Chemistry, Volume 264, No. 26, issued 15 September 1989, O. P. Peoples et al., "Poly-beta-hydroxybutyrate (PHB) Biosynthesis in Alcaligenes eutrophus H16", pages 15298-15303, especially page 15301.	1, 2, 5, 6, 9-11, 16-18
Y	Science, Volume 234, issued 24 October 1986, A. M. Lloyd et al., "Transformation of Arabidopsis thaliana with Agrobacterium tumefaciens" pages 464-466, see entire article.	13, 15, 17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 AUGUST 1992	Date of mailing of the international search report 25 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHARLES RORIES Telephone No. (703) 308-1120

Form PCT/ISA/210 (second sheet)(July 1992)*